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The Roles of a Probiotic Exopolysaccharide in Breast Cancer

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THE ROLES OF A PROBIOTIC EXOPOLYSACCHARIDE IN BREAST CANCER

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%	Percent
5FU	5-Fluoracil
A450	Absorbance at 450nm
ABC	ATP-binding Cassette Transporter
aGvHD	Acute Graft-Versus-Host Disease
AhR	Aryl Hydrocarbon Receptor
AI	Aromatase Inhibitor
AKT	Akt Serine/Threonine Kinase
ALDH1	Aldehyde Dehydrogenase 1
ANOVA	Analysis of Variance
BAK	Bcl-2 Homologous Antagonist/Killer
BCP	1- bromo-3-chloropropane
BCSCs	Breast Cancer Stem Cells
BMDCs	Bone Marrow Derived Dendritic Cells
BMI	Body Mass Index
bp	Base Pair
BRCA1/2	Breast Cancer Gene 1/2
BSA	Bovine Serum Slbumin
C	Celsius

CaCl2	Calcium Chloride
CD	Cluster Differentiation
CDH1	Cadherin 1
CDK4/6	Cyclin-Dependent Kinase 4/6
cDNA	Complementary DNA
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CTX	Cyclophosphamide
d	Day
DCC	Deleted in Colorectal Cancer
DES	Diethystilbestrol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
DNAse	Deoxyribonuclease
dNTPs	Deoxyribonucleotide Triphosphate
E1	Estrone
E2	17β-estradiol
E3	Estriol
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EHS	Engelbreth-Holm-Swarm Tumor

EMT	Epithelial-to-Mesenchymal Transition
EPS	Exopolysaccharide
ER	Estrogen Receptor
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ERK	Extracellular-Signal Regulated Kinase
EtOH	Ethanol
FACS	Flow-Activated Cell Sorting
FBS	Fetal Bovine Serum
FCCP	Carbonyl Cyanide-p-trifluoromethoxyphenylhydrazone
FFARs	Free Fatty Acid Receptors
FGF	Fibroblast Growth Factor
FMT	Fecal Microbiota Transplant
FOXP3	Forkhead Box P3
FRX	Farnesoid X Receptor
GIT	Gastrointestinal Rract
GNRH	Gonadotropin-Releasing Hormone
GPCRs	G Protein Coupled Receptors
h	Hour
HDM	House Dust Mite
hEGF	Human Epidermal Growth Factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HER2	Human Epidermal Growth Factor Receptor 2

HIF	Hypoxia-Inducible Factor
HPRT	Hypoxanthine-guanine Phosphoribosyltransferase
HRP	Horseradish Peroxidase
IC50	Inhibitory Concentration at 50%
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IF	Immunofluorescence
IFN-α/β	Interferon alpha/beta
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IHC	Immunohistochemistry
IkBα	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor, alpha
IKKα/ß	Inhibitor of Nuclear Factor-κB (IκB) Kinase alpha/beta
IL	Interleukin
ip	Intraperitoneal
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRF1	IFN Regulatory Factor 1
JAK1	Janus Kinase 1
JNK	c-Jun N-Terminal Kinase
KCL	Potassium Chloride
kDa	Kilodalton
KO	Knockout
L	Liter

LAB	Lactic Acid Bacteria
LB	Luria Bertani Broth/Agar
LC-MS	Liquid Chromatography–Mass Spectrometry
LCA	Secondary Bile Lithocholic Acid
LPA	Lysophosphotidic Acid
LPAR	Lysophosphatidic Acid Receptor 1
LPS	Lipopolysaccharides
M	Molar
MAPK	Mitogen-Activated Protein Kinase
MaSCs	Mammary Stem Cells
MAVS	Mitochondrial Antiviral Signaling Protein
MD2	Myeloid Differentiation Factor 2
mg	Milligram
MgCl2	Magnesium Chloride
MHC I	Major Histocompatibility Complex (MHC) Class I
Min(s)	Minute(s)
miRNAs	MicroRNAs
mL	Milliliter
mM	Millimolar
MMP-9	Matrix Metalloproteinase-9
MRI	Magnetic Resonance Imaging
mTOR	Mammalian Target of Rapamycin

MyD88	Myeloid Differentiation Primary Response 88
Na3VO4	Sodium Orthovanadate
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NaOH	Sodium Hydroxide
NF-κB	Nuclear Factor-κB
NK	Natural Killer
nM	Nanomolar
nm	Nanometer
OCR	Oxygen Consumption Rate
OD	Optical Density
PALB2	Partner and Localizer of BRCA2
PAMPs	Patterns Associated with Pathogenic Microorganisms
PARP-1	Poly [ADP-ribose] polymerase 1
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD1/PD-L1	Programmed Cell Death Protein 1/Programmed Death-Ligand 1
PE	Phycoerythrin
PEI	Polyethylenimine
PER	Glycolytic Proton Efflux Rate
pg	Picogram
PI-3K	Phosphoinositide 3-Kinase

PMSF	Phenylmethylsulfonyl Fluoride
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homolog
PXR	Pregnane X Receptor
QPCR	Quantitative Polymerase Chain Reaction
RIG-I	Retinoic Acid-Inducible Gene I
RNA	Ribonucleic Acid
RNA-SEQ	RNA-Sequencing
RNAi	RNA Interference
RNAse	Ribonuclease
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute Medium
RQ	Relative Quantification
rRNA	Ribosomal RNA
RT	Reverse Transcriptase
RT-PCR	Real-Time Polymerase Chain Reaction
SCBi	Non-Targeting Scrambled Control siRNA
SCFAs	Short Chain Fatty Acids
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Sodecyl-Sulfate Polyacrylamide Gel Electrophoresis

SEM	Standard Error of Mean
SERMs	Selective Estrogen Receptor Modulators
sgRNA	Single Guide RNA
siRNA	Small Interfering RNA
STAT1	Signal Transducer and Activator of Transcription 1
STAT3	Signal Transducer and Activator of Transcription 3
STK11	Serine/Threonine Kinase 11
SYK	Spleen Tyrosine Kinase
TAAR	Trace Amine-Associated Receptors
TBST	Tris Buffered Saline with Tween 20
TGF-β	Transforming Growth Factor beta
TGR5	Takeda G-Protein Coupled Receptor
Th17/Th1	T Helper 17/ T Helper 1
TLR4	Toll-Like Receptor 4
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor alpha
TNFRI	Tumor Necrosis Factor Receptor 1
TP53	Tumor Protein P53
TPA	Tissue Plasminogen Activator
Tregs	Regulatory T Cells
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon-beta
U.S.	United States

UPS	Ubiquitin–Proteasome System
UV	Ultraviolet
V	Volt
VEGF	Vascular Endothelial Growth Factor
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5 Carboxanilide
γPGA	Gamma-Polyglutamic Acid
µg	Microgram
µm	Micrometer
µM	Micromolar

ABTRACT

Breast cancer is the most common malignancy worldwide. Approximately 85% of breast cancer arises *de novo* in people with no genetic or familial history of breast cancer, suggesting a role for environmental factors in breast cancer development. Recent studies have associated many well-known risk factors for breast cancer with dysbiosis (an aberrant stage of the microbiome). Thus, an important question emerges: how do bacteria within the human body modulate breast cancer? Some initial studies suggest that they could regulate growth of breast cancer via modulation of estrogen metabolism, the immune system, and secretion of carcinogenic metabolites.

The Knight Laboratory focuses on the commensal gut bacterium *Bacillus subtilis*, and has isolated and studied its exopolysaccharide (EPS). Systemic administration of EPS is able to protect mice against a number of T-cell mediated inflammatory diseases, via modulation of TLR4 signaling on myeloid cells to generate anti-inflammatory effects. Although EPS has been proposed as treatment for inflammatory disease, and consumption of *B. subtilis* as a probiotic or in fermented food is considered safe, it was virtually unknown whether *B. subtilis* or *B. subtilis*-derived EPS affects breast cancer.

In this dissertation, we found that *B. subtilis* EPS treatment inhibited the proliferation of some breast cancer cell lines *in vitro,* by inducing cell death or G1 cell cycle arrest in a TLR4-independent manner. However, EPS *in vivo* significantly enhanced xenograft T47D tumor growth in immunocompromised mice. EPS also enhanced cell migration, increased survival of

cancer stem cells, and led to resistance in long-term *in vitro* treatment (months vs days), clueing in how EPS may promote tumor growth *in vivo*. From RNA-SEQ analysis, EPS activated the canonical NF-kB pathway within minutes and the STAT1 pathway within hours. Using CRISPR, siRNA, and chemical inhibitors, we discovered that a novel interaction between IKKβ and STAT1 phosphorylation is critical for EPS to modulate growth of EPS sensitive breast cancer cells. Although many questions remain, this study is the first to investigate the effect of *B. subtilis* EPS on breast cancer and has led to a number of novel, unexpected findings. As the role of the microbiome and its metabolites in cancer is for the most part underexplored, a deeper understanding of how commensal bacteria and microbial products influence breast cancer will lead the way to better prevention strategies and novel therapies for women with breast cancer.

CHAPTER ONE

REVIEW OF LITERATURE

Normal Human Breast Anatomy and Development

Anatomy and Function of the Breast

One of the distinctive traits separating mammals from the rest of the animal kingdom is the presence of mammary glands (breasts), which have evolved from apocrine sweat glands (Macias & Hinck, 2012; Oftedal, 2002). The mammary gland is a unique secretory organ that produces milk to feed newborn offspring. In human, breast tissues located atop the pectoralis major muscle, growing from the nipple deep into a mammary fat pad composing of adipocytes, fibroblasts, infiltrated vascular endothelial cells and immune cells (Alex et al., 2020; Macias & Hinck, 2012). The mature breast is comprised of 15-20 lobes of branched tubuloalveolar glands. The gland epithelium is a bi-layered structure comprising of luminal cells which form the ducts and secretory alveoli, and the basal myoepithelial cells which surround the luminal cells and provide contraction force to squeeze milk secreted by the alveolar cells (Macias & Hinck, 2012). Secreted milk is then drained into the major lactiferous ducts and sinus, and released through the nipple orifice.

Human Breast Development

Embryonic breast development begins approximately at the 5th week of gestation, with the formation of the “milk lines” running from the axilla to the groin

(Anbazhagan et al., 1991; Gusterson, 2012). The milk lines then give rise to a single pair of placodes that mark the location of the breasts (Howard & Gusterson, 2000). Local epithelial/mesenchymal interactions develop the placodes into primary breast buds, with the formation of ductal lumens and the nipple structure (Gusterson, 2012; Kratochwil, 1986; Macias & Hinck, 2012; Robinson et al., 1999). At birth, the mammary gland is a rudimentary ductal system, albeit with secretory capacities upon stimulation (Anbazhagan et al., 1991; Macias & Hinck, 2012).

A few months after birth, breast buds regress as hormones subside. They then grow proportionally with the rest of the body, displaying no sex differences. At thelarche, females experience extensive proliferation of breast and adipose tissues under the influence of estrogen and progesterone (from the ovary), and growth hormones (from the pituitary glands) (Macias & Hinck, 2012). Cyclic hormone fluctuation due to menstruation can stimulate the mammary glands to grow some, but their full maturation capable of milk production only occurs with pregnancy. Pregnancy produces estrogen, prolactin and progesterone, which collaboratively induce tremendous mammary gland proliferation, maturation and alveologenesis to prepare for lactation (Alex et al., 2020; Macias & Hinck, 2012). By late pregnancy, breast glands expand into the fat pad and display secretory activity, along with increased vascularization. After birth, milk secretion is induced by baby's suckling along with the rapid decrease of estrogen and progesterone (Alex et al., 2020). At weaning, milk stagnates within the mammary ducts and initiates the process of involution. Post-lactational involution starts with the apoptosis of milk-producing alveolar cells, followed by massive tissue remodeling to bring the breast back to its

adult virgin state. In contrast, age-related involution of the breast involves replacing the glandular mammary epithelium and its connective tissues with fat (Macias & Hinck, 2012).

Overall, our knowledge of human breast development is surprisingly limited. Gusterson and Stein pointed out that there were only less than 20 primary publications containing significant information on human breast development, mainly due to challenges with tissue acquisition and ethical concerns (Gusterson, 2012). Much of our knowledge on breast development is derived from murine models. Although many researchers use the mouse mammary glands as a model system, differences exist between human and mouse mammary glands in term of architectures, hormones, immune system, and physiology. One important distinction is that sexual dimorphism of the mammary glands is achieved during embryogenesis in mice, while human mammary glands undergo comparable development until puberty (Macias & Hinck, 2012). Lack of knowledge on the human side also makes it difficult to interpret and draw analogies from mouse data (Gusterson, 2012). Thus, caution is advised when translating murine findings to human physiology. It is critical that we continue expanding our understanding of human breast development, as it would also shed insight into pathology of breast diseases and aid in the discovery of novel therapies.

Breast Cancer Epidemiology

Breast cancer is the most common cancer worldwide, accounting for 11.7% of all new cancer cases in 2020 (Sung et al., 2021). In the U.S., the American Cancer Society estimates that 31% of new cancers diagnosed in women in 2022 (approximately 287,850 cases) will be breast cancer (Siegel et al., 2022). As of January 2019, an estimate of more than 3.8 million women living in the U.S. have a history of breast cancer, 64% of which are over 65 years old while only

7% are younger than 50 (Miller et al., 2019). Breast cancer has such high prevalence that the lifetime risk of developing invasive cancer is about 1 in 8 women and 1 in 833 men (Howlader N; Siegel et al., 2022). Since the risk of breast cancer is much lower for men compared to women, men constitute less than 1% of all breast cancer cases in the U.S (Siegel et al., 2022). The incidence of breast cancer rates rose rapidly during the 1980s and 1990s, largely due to increased mammography screening (American Cancer Society, 2019; Breen et al., 2011). The rates then began decreasing, with a sharp drop in invasive breast cancer rate between 1999-2004, attributed to the decrease of Hormone Replacement Therapy following the 2002 Women's Health Initiative study linking use of hormones during menopause with increased risk of breast cancer (American Cancer Society, 2019; Coombs et al., 2010; Ravdin et al., 2007). Since the mid-2000s, the incidence for invasive breast cancer has been steadily increasing by about 0.5% per year, partly due to the recent declines in fertility rate (average number of births per woman) and increases in body mass index (excess weight/obesity) (American Cancer Society, 2019; Pfeiffer et al., 2018).

Breast cancer is the second-leading cause of cancer-related death in the U.S., behind only lung cancer. It is estimated that 43,250 women and 530 men living in the U.S. will die of breast cancer in 2022 (Siegel et al., 2022). Fortunately, the overall death rate from breast cancer has drastically dropped by 40% in the years 1989-2017 (American Cancer Society, 2019; Siegel et al., 2022). Although this decrease in death rate has slowed slightly in recent years, breast cancer mortality continues to decline by about 1% per year from 2013-2019 (American Cancer Society, 2019; Siegel et al., 2022). Early detection and improved treatments both contributed to the observed decline in breast cancer mortality over the past decades (Berry et al., 2005). For women

diagnosed with breast cancer in the U.S., relative survival is excellent, ranging from 91% at 5 years after diagnosis to 80% after 15 years (American Cancer Society, 2019). Stage at diagnosis, or how far the tumor has spread, has significant impact on patient survival, as localized disease has 99% 5-year survival, while metastatic disease only has 27% (American Cancer Society, 2019). Although patient survival also varies greatly by tumor subtype, a study in 2018 reported that 4-year survival was 95% for patients with stage I (localized) breast cancer across all tumor subtypes (Howlader et al., 2018). This further highlights the importance of detecting breast cancer early in order to maximize treatment efficacy and survival.

It is of note that breast cancer mortality is significantly higher in developing countries compared to developed countries (15.0 vs 12.5 per 100,000 cases), with sub-Saharan African regions ranking in the world deadliest (60% 5-year survival compared to 91% in the U.S.) (Allemani et al., 2018; Sung et al., 2021). The low survival rate in developing countries is attributed to late stage presentation (77% sub-Saharan cases presented at stage III/IV at diagnosis compared to <35% of U.S. cases), which resulted from the lack of mammography screening programs, healthcare structures and resources (American Cancer Society, 2019; Jedy-Agba et al., 2016; Sung et al., 2021). Even within a developed nation such as the U.S., cancer disparity is evident between race and ethnicity. In the period between 1970 to 2010, 5-year relative survival increased from 76% to 92% for white women, but only went from 62% to 83% for black women (American Cancer Society, 2019). Lower survival here has been associated with poverty, less education and lack of health insurance (American Cancer Society, 2019; Ellis et al., 2018; Singh & Jemal, 2017). Aside from socioeconomic factors, recent findings suggest that African Americans may be predisposed to more aggressive breast tumors, which can influence tumor

behavior and outcome (Rojas & Stuckey, 2016). Thus, there may be multiple variables contributing to differences in breast cancer incidence and mortality observed across different populations.

Breast Cancer Pathogenesis

Histopathological observations modelled breast cancer progression through defined stages over long periods of time, starting from ductal hyperproliferation to in situ carcinoma, invasive carcinoma, and metastatic disease (Allred et al., 2001; Polyak, 2007; Sgroi, 2010; Wellings & Jensen, 1973). Initiation of breast cancer is thought to occur by transforming events (genetic or epigenetic) within a single breast epithelial cell, which confers a growth advantage and eventually gives rise to a cell with proliferative capacity (benign lesion) (Wellings & Jensen, 1973; Wellings et al., 1975). Benign lesions such as atypical hyperplasias and in situ carcinomas are rather common (Allred et al., 2001). These cells have lost control over proliferation, but have yet to acquire the ability to invade through the basement membrane and metastasize to distant organs. Although these benign lesions possess the potential to evolve into malignant tumors, the majority of them will not progress further (Allred et al., 2001). The mechanisms that promote progression of breast cancer from a benign to a malignant state are poorly understood.

Traditionally, tumor progression is thought to be driven by the accumulation of molecular alternations along with clonal selection and expansion of specific tumor cells. However, comprehensive studies have failed to identify stage-specific gene signatures in the epithelial compartment of breast tumors (Chin et al., 2004; Ma et al., 2003; Porter et al., 2003; Porter et al., 2001; Yao et al., 2006). This suggests that other factors may be driving breast cancer progression. Recent studies demonstrate that active interactions between the neoplastic breast

epithelial cells and non-neoplastic cells within the tumor microenvironment (fibroblasts, myoepithelial cells, endothelial cells, and immune cells) are important in driving breast cancer initiation and progression (Sgroi, 2010). Manipulation of the breast stromal microenvironment was shown to have profound influences on tumor growth, invasion, and metastasis (Cheng et al., 2005; Elenbaas & Weinberg, 2001; Maffini et al., 2004; Polyak, 2007; Price et al., 1990). Although dramatic gene-expression changes were found in the non-neoplastic myofibroblastic cells and myoepithelial cells of the breast tumor microenvironment, there were no clonal genetic alternations within stromal cells (Allinen et al., 2004). Thus, epigenetic modifications in the non-neoplastic stromal cells are thought to be responsible for the changes in the breast tumor microenvironment (Hu et al., 2005). Taken together, the latest findings point to a combination of changes in epithelial tumor cells and factors in the microenvironment collectively working together to drive breast cancer invasion and metastasis.

The origin of breast cancer is an ongoing debate. Currently there are 2 prevailing models to explain breast cancer oncogenesis: the stochastic model (also called the clonal evolution model) and the cancer stem cell model (Sgroi, 2010). Classically, the stochastic model proposes that random mutations and transformations can originate in any breast epithelial cell, allowing it to proliferate uncontrollably. Genetic instability within the original clone of cells then gives rise to additional genetic variations among different clones, allowing for expansion of more aggressive clones as the tumor progresses (Nowell, 1976). Overtime, cancer cells can also coevolve in their microenvironment and accumulate more genetic alterations, resulting in cellular heterogeneity within a tumor (Polyak et al., 2009). In contrast, the cancer stem cell model hypothesizes that all neoplastic cells in the tumor are derived from a small population of cells

referred to as cancer stem cells. These cancer stem cells originate from normal mammary stem cells or progenitors that have been transformed by a genetic alteration, resulting in aberrant differentiation giving rise to phenotypic heterogeneity in breast tumors (Dontu et al., 2003; Polyak, 2007; Wicha et al., 2006). There is evidence to support both models, and it is possible that early tumor progression may require elements from both models, i.e. clonal selection of cancer stem cells (Sgroi, 2010). More research is needed to identify the model which best explains tumor behavior in patients and develop novel strategies to target the different drivers in subpopulation of cells (Polyak, 2007).

Breast Cancer Subtypes

Investigation and therapeutic strategies to cure breast cancer have been challenging due to its heterogeneous nature. It is comprised of very distinct entities in term of clinical and molecular characteristics. Based on gene expression profiling, breast cancer is classified into at least 5 intrinsic molecular subtypes (Luminal A, Luminal B, HER2 enriched, Triple-negative\Basal-like, Claudin-low) and a normal breast-like group (Eroles et al., 2012; Perou et al., 2000; Sotiriou et al., 2003). Each subtype of breast cancer has its own prognosis, treatment response, presumed behavior and pathogenesis.

Luminal A Breast Cancer

Luminal A is the most common breast cancer subtype, accounting for 50-60% of total breast cancer cases (Al-thoubaity, 2020; Eroles et al., 2012). It is characterized by expression of estrogen and progesterone receptors, absence of overexpression of the epidermal growth factor receptor-2 (ER+/PR+/HER2-), and generally expresses genes activated by the ER transcription factor. Luminal A tumors express low level of Ki67 and other proliferation markers (Perou et al.,

2000; Sorlie et al.). They tend to be low grade and have good prognoses (Hennigs et al., 2016). Patients with this subtype have the highest 5-year survival rate (94.4%) and lower relapse rate (27.8) than other subtypes (Howlader N; Kennecke et al., 2010). Recurrence tends to follow a distinct pattern, with higher rate of bone metastases. Treatments for this subtype include agents that competitively inhibit binding of estrogen to the ER (tamoxifen and fulvestrant) or prevent synthesis of estrogen by inhibiting the aromatase enzyme [aromatase inhibitors (AI)] (Guarneri & Conte, 2009).

Luminal B Breast Cancer

Luminal B constitutes 10-20% of all breast cancer cases (Al-thoubaity, 2020; Eroles et al., 2012). These tumors express ER and/or PR, but they may or may not overexpress HER2 (ER+/PR+/HER2+/-). They also express high levels of the proliferative marker Ki67, which distinguishes them from Luminal A tumors (Cheang et al., 2009). Among luminal tumors, luminal B tumors tend to be more aggressive, with higher histological grade, proliferative index, and worse prognosis (Eroles et al., 2012; Fan et al., 2006; Loi et al., 2007). Compared to the luminal A subtype, patients with luminal B have slightly lower 5-year survival (90.7% vs 94.4%) and different pattern of relapse (Howlader N; Kennecke et al., 2010). Main treatment options include the use an AI or tamoxifen, cytotoxic chemotherapy, and anti-HER2 therapy if applicable (Burguin et al., 2021).

HER2 Enriched Breast Cancer

HER2 enriched tumors represent 15-20% of breast cancer cases (Al-thoubaity, 2020; Eroles et al., 2012). They are characterized by amplification of the ERBB2 gene and hyperactivity of its associated signaling pathways (MAPK and PI-3K). These tumors are highly

proliferative and aggressive, with high histological grade and poor prognosis (Eroles et al., 2012; Hennigs et al., 2016). The 5-year survival for HER2 subtype is about 84%, with certain subgroup within the HER2+ subtypes having even worse survival (12% 10-year survival) (Howlader N; Staaf et al., 2010). As a result of overexpression of the HER2 protein, these tumors are highly sensitive to anti-HER2 therapy in combination with cytotoxic chemotherapy (Eroles et al., 2012; Parker et al., 2009). In the last decade, improvement in anti-HER2 therapy has significantly increased survival for patients with HER2+ breast cancer (Eroles et al., 2012; Gianni et al., 2011; Slamon et al., 2001).

Basal-like Breast Cancer

The basal-like subtype accounts for 10-20% of breast tumors (Al-thoubaity, 2020; Eroles et al., 2012). It is aptly called triple-negative subtype, due to the absence of ER, PR and HER2 expression. These tumors tend to express genes present in normal breast myoepithelial cells (Eroles et al., 2012). Many of them carry p53 mutations or BRCA1 germline mutations (Sorlie et al.; Tibshirani et al., 2003). Patients with basal-like tumors are predominantly women of African origin and tend to present at younger age with larger tumors, high histological grade, and lymph node involvement at diagnosis (Bosch et al., 2010). Overall, women with basal-like tumors have very poor prognosis and a high relapse rate (Dent et al., 2007; Eroles et al., 2012; Sorlie et al.). The 5-year survival for patients with these tumors is the poorest among the subtypes, at 77% (Howlader N). Women with basal-like breast cancer are treated with cytotoxic chemotherapy regimens due to the lack of treatment targets. Currently, chemotherapy along with PARP-1 inhibitors seems to be the most promising option (Fong et al., 2009).

Claudin-low Breast Cancer

The claudin-low subtype was first identified in 2007, after other subtypes (Herschkowitz et al., 2007). It accounts for 12-14% of breast cancer (Eroles et al., 2012). This subtype is characterized by low expression of tight junction genes such as claudins and E-cadherin (Prat et al., 2010). It shares characteristics with basal-like tumors, expressing low level of HER2, ER/PR and other luminal genes. Intriguingly, these tumors express high levels of genes that promote epithelial to mesenchymal transition and tumor immune response (Prat et al., 2010). Similar to basal-like tumors, patients with claudin-low tumors have poor long-term prognosis, partly due to the lack of targeted treatments and poor response to neoadjuvant chemotherapy (Prat et al., 2010; Prat & Perou, 2011).

Normal Breast-like Breast Cancer

The normal breast-like subtype is considered rare, found only in 5-10% of breast tumors (Eroles et al., 2012). These tumors lack expression of ER, PR, and HER2, and express genes normally present in adipose tissues. Overall, they are poorly characterized, and have been confused with normal breast samples and fibroadenomas (Perou et al., 2000). Prognosis for women with these tumors is an intermediate between luminal and basal-like subtypes (Eroles et al., 2012). They generally respond poorly to chemotherapy. Given the rarity of these tumors, there are doubts regarding their existence and any clinical significance they may have (Eroles et al., 2012; Weigelt et al., 2010).

Challenges in Breast Cancer and Breast Cancer Stem Cells

Current Challenges in Breast Cancer and New Developments

Over the last 4 decades, early detection and improved treatments have drastically dropped breast cancer mortality by 40%, and the survival rate is very high for most patients (Siegel et al., 2021, 2022). However, challenges remain as many patients still have relapse and die as a result of recurrences, metastases and/or drug resistance (Sin & Lim, 2017; Zeng et al., 2021). It is estimated that up to 30% of early stage breast cancer will recur, with most presenting as metastases (Burguin et al., 2021). The 5-year cancer-specific survival rate for patients with breast cancer across all subtypes and stages is 90.3%, but this number drops to 29% for patients with metastatic disease and decreases to 12% for basal-like metastatic breast cancer (Burguin et al., 2021; Siegel et al., 2022). Patients with metastatic breast cancer are reported to have 20-30 months median survival (Caswell-Jin et al., 2018). These statistics highlight our current challenges in treating breast cancer, particularly in dealing with drug resistance, recurrence, and metastasis.

Recent developments have revealed exciting new tools to treat breast cancer. Since PI-3K/AKT/mTOR signaling pathway and the cell cycle regulators Cdk4 and Cdk6 play critical roles in resistance to endocrine therapy, multiple therapeutic strategies are being examined to inhibit these pathways (Burguin et al., 2021). For HER2+ disease, new treatment strategies are under development, including new HER2 targeting antibodies, new tyrosine kinase inhibitors, and new antibody-drug conjugates (Escrivá-de-Romaní et al., 2018). Immunotherapies such as checkpoint inhibitors using anti-PD1/PD-L1 antibodies and peptide vaccines are also being investigated, particularly for triple-negative breast cancer which lacks targeted treatment

(Bianchini et al., 2016). Other targeted therapies include PARP inhibitors, histone deacetylase inhibitors, VEGF and EGFR inhibitors (Burguin et al., 2021).

Breast cancer is a complex disease, difficult to manage due to its inherent heterogeneity across the molecular, phenotypic, and functional features within a patient's tumor and across different patients (Koren & Bentires-Alj, 2015). Tumor heterogeneity across patients may be explained by intrinsic molecular subtypes of breast cancer, while inter-tumor heterogeneity can be explained with the cancer stem-cell hypothesis (Rabinovich et al., 2018). As discussed above, increasing evidence points to the existence of a subpopulation of breast cancer stem cells (BCSCs) within a tumor. These cells are thought to drive initiation and progression of breast cancer. They are resistant to conventional therapies and can repopulate the tumor, leading to frequent recurrences and metastatic spread to vital organs (Kakarala & Wicha, 2008). Thus, novel strategies are needed to target BCSCs as well as the bulk breast cancer cells to completely eradicate breast cancer and prevent relapse.

The Cancer Stem-Cell Hypothesis and the Origin of BCSCs

The cancer stem-cell hypothesis postulates that there is a small population within the tumor displaying stem-like characteristics, which is responsible for tumor initiation, treatment resistance and recurrence (O'Flaherty et al., 2012). In accordance with this hypothesis, recent data supported the existence of a small population of undifferentiated BCSCs with self-renewal and full differentiation capacity within the tumor (Khan et al., 2021; Scioli et al., 2019). Moreover, this subpopulation of cancer stem cells is able to repopulate and reproduce the full heterogeneity of the original tumors, as observed in leukemia and other types of cancer (Kakarala & Wicha, 2008; Lapidot et al., 1994).

Although controversial, one plausible hypothesis proposes that BCSCs originate from mammary stem cells (MaSCs) (Sin & Lim, 2017). MaSCs are long-lived, self-renewing, and multipotent stem cells residing in breast tissues that can give rise to both the luminal and basal epithelial lineages (Ercan et al., 2011; Koren & Bentires-Alj, 2015; Stingl et al., 2001). These MaSCs grow within a stem cell niche and respond to environmental cues such as estrogen, Wnt, Hedgehog, Notch, TGF-β and growth factor signaling pathways (Ercan et al., 2011; Khan et al., 2021; Villadsen et al., 2007). MaSCs are critical for the physiological functions of the breast, as proper response by MaSCs leads to the dynamic changes in the mammary glands seen during puberty, menstruation and pregnancy (Ercan et al., 2011). Due to their inherent stem cell characteristics, MaSCs or progenitors may acquire mutations leading to transformation and tumorigenesis, giving rise to BCSCs with dysregulated mammary stem cell characteristics (Bao et al., 2015; Kakarala & Wicha, 2008; Khan et al., 2021; Lim et al., 2009; Liu et al., 2014; Tharmapalan et al., 2019; Zhang et al., 2020).

Identification Markers and Signaling Pathways Critical for BCSCs

Various study estimated BCSCs to comprise 0.1-1.0% of the tumor bulk (Honeth et al., 2008; Rabinovich et al., 2018; Ricardo et al., 2011; Tanei et al., 2009; Zhang et al., 2020). These BCSCs are identified as tumor-initiating cells expressing aldehyde dehydrogenase 1 ($ALDH1^+$) and/or $CD44^+/CD24^{-/low}$ (de Beça et al., 2013). The evidence supporting these markers were first reported by Al-Haji *et al* in 2003. In this study, as few as 100 cells expressing the $CD44^+/CD24^-/Lineage^-$ phenotype in human breast tumors were able to form tumors in immunodeficient mice with similar heterogeneity to that of the original tumor, while tens of thousands of cells with other phenotypes failed to form tumors (Al-Hajj et al., 2003). Moreover,

these $CD44^{+}/CD24^{-}$ BCSCs displayed stem-like properties *in vitro*, including self-renewal, differentiation along mammary epithelial lineages, clonal nonadherent spherical clusters (mammosphere formation), extensive proliferation, and resistance to chemotherapy (Fillmore & Kuperwasser, 2008; Ponti et al., 2005).

Several signaling pathways were identified as critical regulators of BCSCs. Unsurprisingly, embryonic developmental pathways such as Notch, Wnt/β-catenin, Hedgehog and Hippo played a key role in BCSC. Since these embryonic developmental pathways regulated self-renewal and differentiation of normal mammary stem cell, dysregulation or aberrant activation of these pathways transformed normal stem cells into BCSCs and led to tumorigenesis (Khan et al., 2021; Pires et al., 2016). In addition, inflammation-related pathways (NF-κB and STAT3), BCSC microenvironment and tumor hypoxia (TGF-β and HIF), proliferative pathways (HER2 and PI3K/AKT) and the loss of tumor suppressor gene (BRCA1) all contributed to the stemness properties of BCSCs (Khan et al., 2021; Pires et al., 2016). Finally, several microRNAs and long non-coding RNAs also modulated genes associated with stem cell pathways to regulate self-renewal and survival of BCSCs (Khan et al., 2021; Wu et al., 2019; Yu et al., 2010; Yu et al., 2007; Zeng et al., 2021; Zhang et al., 2014; Zhang et al., 2020; Zhou et al., 2016).

BCSCs in Treatment Resistance and BCSC Targeted Therapy

BCSCs correlated with poor prognosis and played critical roles in tumorigenesis, metastasis, recurrence, and resistance to conventional therapy (Bartucci et al., 2015; Charafe-Jauffret et al., 2009; Creighton et al., 2009; W. Li et al., 2017; Palomeras et al., 2018; Rabinovich et al., 2018; Ricardo et al., 2011; Yin & Glass, 2011; Zhang et al., 2020). Recent data specifically identified BCSCs as the key drivers responsible for first-line treatment

resistance, leading to treatment failure and cancer relapse (Diehn et al., 2009; Khan et al., 2021). The BCSC tumor subpopulation displayed inherent resistance to conventional chemotherapy due to its high expression of drug transporters, cell fate plasticity and ability to enter a quiescence state, resistance to oxidative stress, DNA damage, and/or pro-apoptotic agents (Brown et al., 2017; Palomeras et al., 2018; Singh et al., 2013; Yin & Glass, 2011; Zeng et al., 2021).

Since conventional therapies failed to target BCSCs, novel strategies are needed to eradicate these resistant cells. One rational approach is to inhibit the self-renewal pathways that BCSCs depend on, using Notch inhibitors (γ-secretase inhibitors), Wnt monoclonal antibodies, Hedgehog small molecule inhibitors, Hippo/mevalonate pathway inhibitors, HER-2 inhibitors, and PI3K/AKT inhibitors (Khan et al., 2021; Zeng et al., 2021). Another strategy is to target the BCSC microenvironment via TGF-β inhibitors and CD44v6 monoclonal antibody (Zeng et al., 2021). Additional approaches include targeting the DNA damage response, ABC drug transporters, Cdk4/6 inhibitors, miRNAs, and the ubiquitin–proteasome system (UPS) (Khan et al., 2021; Zeng et al., 2021). Lastly, immunotherapy targeting PD-L1 and other novel BCSC antigens is also being investigated as a potential treatment avenue (Khan et al., 2021). Although a number of these stem cell therapies showed encouraging results in clinical trials, none have yet to be approved (Khan et al., 2021). As toxicities and off target effects on normal stem cells remain as major concerns, research is ongoing to find more effective and safer therapy against BCSCs.

Breast Cancer Risk Factors and Prevention

To understand the impact of risk factors, some terminologies need to be defined. Absolute risk is defined as the likelihood of being diagnosed with breast cancer over a certain

time period, while lifetime risk is the absolute risk of being diagnosed over the period of birth to death. Relative risk, on the contrary, compares the absolute risk among people with risk factors to the risk of people without them. Relative risk higher than 1.0 signifies that the risk of breast cancer is higher for those with the risk factor, while relative risk lower than 1.0 indicates lower breast cancer risk or protective effects (American Cancer Society, 2019).

For breast cancer, the highest risk factors are sex and age. In fact, a woman living in the U.S. has a 12.9% absolute risk of being diagnosed with breast cancer in her lifetime (or 1 out 8 women), while the lifetime risk for men is 100 times less, standing at 1 out of 833 men (Howlader N; Siegel et al., 2022). Like other cancers, the absolute risk of breast cancer increases with age (nearly doubles from 12 out of 10,000 women ages 40-44 to 23 out of 10,000 women ages 50-54 being diagnosed in the next year) (American Cancer Society, 2019). In addition, a number of risks factors are known to increase the incidence of breast cancer, including family history, genetic mutations, hormone exposures, smoking and alcohol. These will be discussed in details below. On the other hand, factors such as abortion, wearing bras, and having breast implants have no association with increasing incidence of breast cancer (L. Chen et al., 2014; Couzin, 2003; Leberfinger et al., 2017). An important point to note is that many of the identified risk factors are associated with hormone-receptor-positive breast cancers, while the specific contributions of each factor to the different breast cancer subtypes (triple negative in particular) remain largely elusive. Proper understanding of these risk factors is critical, as it would guide appropriate precautions and prevention strategies.

Familial, Genetic, and Personal Risk Factors

While 85% of breast cancer arises *de novo* in women with no inherited genetic or family history of breast cancer, familial breast cancer still accounts for about 15% of all breast cancer cases (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Shiyanbola et al., 2017). Women and men who have a first-degree relative (parent, child, or sibling) diagnosed with breast cancer have nearly double the risk of breast cancer compared to those who do not. The relative risk increases further for people with more than one first-degree relative affected by breast cancer, or if the affected relative was diagnosed at a younger age, had cancer in both breasts, or was a man (Collaborative Group on Hormonal Factors in Breast Cancer, 2001; Kharazmi et al., 2014). Although having a family history certainly increases one's relative risk of breast cancer, the majority of women with a family history of breast cancer will never develop breast cancer, and those who do, tend to get diagnosed after 50 years old (Collaborative Group on Hormonal Factors in Breast Cancer, 2001).

Similarly, having a family history of ovarian, pancreatic or prostate cancer is also associated with increased breast cancer risk, which suggests underlying germline mutations (Beebe-Dimmer et al., 2015; Mocci et al., 2013). Mutations in the BRCA1 and BRCA2 DNA repair genes are some of the most commonly associated alternations in these cancers. In fact, mutations in BRCA1/2 account for nearly a quarter of all familial breast cancers, and up to 10% of all breast cancers (American Cancer Society, 2019; Tung et al., 2016). Women who carry BRCA1/2 mutations have a 70% absolute risk of developing breast cancer by the age 80, compared to 10% risk of the general population (Kuchenbaecker et al., 2017). Furthermore, breast cancer in these women tends to develop at a younger age (Kuchenbaecker et al., 2017).

Mutations in other genes, such as PALB2, TP53 (Li-Fraumeni syndrome), PTEN (Cowden syndrome), STK11 (Peutz-Jeghers syndrome), and CDH1 (gastric and lobular breast cancer syndrome) are also associated with increased risk of breast cancer (Antoniou et al., 2014; Bagherpour et al., 2018; Sidransky et al., 1992; Tacheci et al., 2021; Tan et al., 2012).

A number of personal factors concerning one's breast tissues has also been associated with increased risk of breast cancer. Namely, women diagnosed with breast cancer have approximately 5% increased risk of developing contralateral breast cancer (the other breast), which can be lowered with adjuvant therapies (Kramer et al., 2019). Women diagnosed with lobular carcinoma in situ are at double the lifetime risk of invasive breast cancer (Rojas & Stuckey, 2016; Rosen et al., 1978). Nonproliferative benign breast lesions (cysts, fibroadenomas) carry little relative risk of breast cancer, while proliferative lesions without atypia (adenosis, intraductal papilloma) double the risk, and proliferative lesions with atypia (atypical ductal hyperplasia, atypical lobular hyperplasia) quadruple the relative risk (Dyrstad et al., 2015). Having dense breast tissue also increases the risk for breast cancer by 1.5 to 2-fold, and makes detection of breast tumors by mammography difficult (Bertrand et al., 2013; Boyd et al., 2007).

Hormonal Exposure Risk Factors

Longer exposure to reproductive hormones is significantly associated with increased incidence of hormone-receptor-positive breast cancer. In particular, risk of breast cancer increases with early menarche (before aged 12), late menopause (after aged 55), high levels of endogenous hormones in postmenopausal women, exposure to synthetic estrogens including diethystilbestrol (DES), recent use of oral hormonal contraceptives and menopausal hormone therapy combining estrogen and progestin (American Cancer Society, 2019; Bassuk & Manson,

2015; Brown & Hankinson, 2015; Collaborative Group on Hormonal Factors in Breast Cancer, 2012; Manson et al., 2013; Mørch et al., 2017; Titus-Ernstoff et al., 1998; Trichopoulos et al., 1972). On the other hand, parity, younger age at first term pregnancy, greater number of children, and longer breastfeeding were found to have protective effects against breast cancer (Clavel-Chapelon & Gerber, 2002; Collaborative Group on Hormonal Factors in Breast Cancer, 2002; Li et al., 2013; Ma et al., 2006; Nichols et al., 2019).

Lifestyle Risk Factors

A number of lifestyle factors carry significant impact on breast cancer risk. Obesity and weight gain have been shown to increase the risk of breast cancer after menopause, which is thought to be due to increased levels of estrogen and insulin from adipose tissues (Jiralerspong & Goodwin, 2016; Picon-Ruiz et al., 2017). In premenopausal women, some studies actually found that excess body weight may protect against breast cancer through a poorly understand mechanism (Picon-Ruiz et al., 2017). Alcohol consumption was shown to increase the risk of breast cancer by 10% per drink per day, particularly for hormone-receptor-positive tumors (Jung et al., 2016; Liu et al., 2015). Heavy smoking in women (particularly before their first pregnancy) and exposure to second hand smoke during childhood slightly increased the risk of breast cancer (Gaudet et al., 2013; White et al., 2017). Exposure to radiation, particularly as high-dose radiation to the chest between ages 10 and 30, increases breast cancer risk for years after exposures (Ehrhardt et al., 2019; Schaapveld et al., 2015). Surprisingly, nightshift work and disruption to the circadian rhythm were also shown to increase breast cancer risk (Jia et al., 2013).

On a positive note, certain diet and exercise can protect against breast cancer. Studies have shown that regular level of physical activities can lower the relative risk of breast cancer by 10-20% through positive effects on energy, hormones, and systemic inflammation (American Cancer Society, 2019; McTiernan et al., 2019; Neilson et al., 2009). As for diets, studies thus far are rather limited and produced conflicting results. However, initial evidence suggests that soy consumption (particularly in Asian women), high levels of fruits, vegetables and dairy/calcium consumption may be associated with a lower risk of breast cancer (Buja et al., 2020; M. Chen et al., 2014; Eliassen et al., 2012; Jung et al., 2013; Y. Wu et al., 2021). More research is needed to determine the timing of exposure and dietary components required to protect against breast cancer, as well as effects on different breast cancer subtypes (American Cancer Society, 2019).

Breast Cancer Prevention Strategies

Breast cancer is preventable, and treatments are highly effective if patients are diagnosed early (Sun et al., 2017). Thus, based on each patient's personal relative risk levels, appropriate screening and prevention may be utilized to monitor and minimize the development of breast cancer. There are three main strategies for breast cancer prevention: prophylactic mastectomy, chemoprevention, and regular mammography screening. Bilateral mastectomy may be applied to women at very high risk of breast cancer, such as those carrying a BRCA1/2 mutation, since it can reduce the risk of breast cancer by more than 90% (Kotsopoulos, 2018). Chemoprevention, or the use of drugs or natural agents to inhibit the development of invasive breast cancer, is mainly achieved through the use of selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene (Sporn, 1976; Sun et al., 2017). The use of SERMs was found to reduce the incidence of invasive hormone-receptor-positive breast cancer by 38% over 10 years

(Cuzick et al., 2013). Another class of anti-estrogens, an aromatase inhibitor (i.e. anastrozole), was also shown to lower the incidence of invasive breast cancer in postmenopausal women (Nelson et al., 2019). Although these chemopreventive drugs have been shown to reduce breast cancer risk, care must be taken to identify which high-risk patients would benefit from these drugs as they are also associated with significant adverse effects. Lastly, mammography and MRI screening are recommended for women of certain ages and/or high-risk groups. Mammography uses a low-dose X-ray to produce high-resolution images of breast tissue, and MRI is used when higher sensitivity is desired. Overall, screening remains the cornerstone of breast cancer prevention and education. Early cancer detection via screening has shown to reduce breast cancer death by up to 40% (Coldman et al., 2014; Tabár et al., 2019). This goes on to show that screening saves lives, and prevention works.

The Microbiome and its Relation to Breast Cancer

Introduction

The Human Microbiome Project is an initiative by the National Institute of Health launched worldwide in 2007, aimed to understand the human microbiome and how it contributes to health and disease (Turnbaugh et al., 2007). Although the terms microbiota and microbiome are often used interchangeably, they carry slightly different meanings. Microbiota refers to the taxonomy and abundance of microbes present in a particular environment, while microbiome points to the expression of microbial genes and their functions by the present microbiota (Ursell et al., 2012). The study of the microbiome first originated from puzzling findings that there were only 20,000-25,000 protein-encoding genes found in the human genome with 99.9% identical expression between individuals (International Human Genome Sequencing Consortium, 2004;

Ursell et al., 2012; Wheeler et al., 2008). In contrast, the number of microbes is approximately equal to the number of their human host cells and their collective genome exceeds that of humans by more than 100-fold (Gill Steven et al., 2006; Sender et al., 2016). The microbiome also presents much greater diversity and variation, being up to 80-90% different from person to person (Gill Steven et al., 2006; Human Microbiome Project Consortium, 2012; International Human Genome Sequencing Consortium, 2004; Ursell et al., 2012). Taken together, the thought emerged that the microbiome may collaborate/coevolve with its human host as a "supra-organism," consequently contributing to traits that distinguish human from other species and make each human his/her own person (Parida & Sharma, 2020; Turnbaugh et al., 2007). The human microbiota is known to have an unique niche at different body sites, with most research attention focused on the skin, mouth, nose, colon and vagina (Parida & Sharma, 2020). Recent studies have reported the presence of a unique microbiota at sites previously thought to be sterile, including the lungs, pancreas, prostate, and breasts (Parida & Sharma, 2020). The microbiome has been shown to play critical roles in normal physiology and to contribute to a number of different diseases including metabolic disorders, inflammatory and autoimmune diseases, and various organ specific cancers (Fernández et al., 2018; Parida & Sharma, 2020; Rea et al., 2018; Selber-Hnatiw et al., 2017). These studies highlight the need to understand how the microbiome functions and how we can manipulate the microbiota to maximize health and treat disease.

Methods to Study the Microbiome

Advances in genomic sequencing technologies in the last decades has enabled the explosion in the study of the microbiome (Turnbaugh et al., 2007). Next Generation Sequencing offers unprecedented high resolution and high throughput analysis of the microbial community

present in a biological sample, bypassing limits of *in vitro* culture (Jovel et al., 2016; Quince et al., 2017). Thus, main approaches for studying the microbiome are 16S ribosomal RNA (rRNA) sequencing and shotgun metagenomics (Laborda-Illanes et al., 2020). Since the 16S rRNA gene is conserved across bacteria and archaea, sequencing of this gene's PCR amplicons allows for the identification and quantification of microbes within a sample (Caporaso et al., 2011; Janda & Abbott, 2007). Although 16S rRNA sequencing is cost-effective and can cover a large number of samples, it has limited taxonomical and functional resolution (Jovel et al., 2016). In contrast, whole-metagenome shotgun sequencing targets all genes from all cells present in a sample, providing information on both taxonomic composition and biological functions (Quince et al., 2017; Sharpton, 2014). Shotgun sequencing offers high resolution and coverage, but it is expensive and requires complex data analysis (Quince et al., 2017). Other challenges to genomic sequencing include contamination during sample collection and processing, sample storage, DNA isolation efficiency, and sequencing error (Fricker et al., 2019).

Additional approaches include RNA sequencing to capture the metatranscriptome, mass spectrometry and nuclear magnetic resonance analysis to characterize the metabolomics and metaproteomics produced by the microbial community in the sample (Laborda-Illanes et al., 2020). Scanning electron microscopy and fluorescence in situ hybridization may be employed to identify and visualize microbes in fixed samples, albeit with limited capacities (Earle et al., 2015; Lukumbuzya et al., 2019). Thus, researchers must select the appropriate methods to analyze the microbiome. Multiple approaches combining metagenomics, metatranscriptomics, metaproteomics and metabolomics may be necessary to create a comprehensive picture of the microbiome of interest (Laborda-Illanes et al., 2020).

The Gut Microbiome

The gut microbiome is the largest, most diverse and well-studied microbiome in the human body. It is estimated that over 70% of all microbes residing in the human body is found in the colon (Sun, 2018). Colonization of the gut with microbes early in life is essential to the development of the immune system and overall health, with some evidence suggesting that microbial exposure begins intrauterine via the mother's placenta (Alpuim Costa et al., 2021; Stinson et al., 2019). The mode of birth also affects the microbiome of newborns, with vaginally delivered babies having the vaginal flora of the mother dominated by *Lactobacillus* and caesarean babies having the skin microbiota dominated by *Staphylococcus* (Stewart et al., 2018). During the developmental phase of the microbiome in the first year of life, breastfeeding is the main factor driving the diversification of the gut microbiome (Stewart et al., 2018). As babies grow, geographical location (urban or rural) and household exposures (living with sibling and furry pets) are important factors for the development of the microbiome. Finally, the microbiome stabilizes at 31 months of age and remains throughout adulthood, with slight modifications stimulated by diet, lifestyle and/or pathologies (Alpuim Costa et al., 2021; Stewart et al., 2018). After 65 years of age, the microbiome becomes less diverse and less stable, resembling a dysbiosis state (Maynard & Weinkove, 2018).

Once fully established, the gut microbiome provides essential functions for the host. Recent evidence suggests that the gut microbiome can reach beyond the intestine and digestion, affecting systemic processes and other organ functions (especially the brain) via its capacities to modulate the gut-brain axis, cardiovascular, neural and immune systems (J. Zhang et al., 2021).

Thus, changes in the gut microbiome are starting to be considered as a contributing factor in many diseases, including breast cancer (Fernández et al., 2018; J. Zhang et al., 2021).

The Breast Microbiome

Although human breast milk is known to carry a distinctive collection of bacteria, breast tissues themselves were presumed to be sterile (Hunt et al., 2011; Urbaniak et al., 2012). Urbaniak et al was the first to discover that the breast actually contained its own unique microbiome, which was confirmed in subsequent studies (Parida & Sharma, 2019a; Urbaniak et al., 2014). The finding that the breast microbiota is distinct from that of other body sites is consistent across breast sample location, pregnancy history, breast cancer history, age, country of origin, and sequencing technologies (J. Zhang et al., 2021). The breast microbiota is more diverse than the vaginal microbiota, but is slightly lower compared to other organ sites such as the gut (Urbaniak et al., 2014). Most studies found *Proteobacteria* and *Firmicutes* to be predominant in the breast tissues, except one, where *Bacteroidetes* dominated (Hieken et al., 2016; Urbaniak et al., 2014; Urbaniak et al., 2016; Xuan et al., 2014). Several access routes are thought to give rise to microbes residing in the breast: translocation from the gut through an endogenous route involving dendritic cells, passage from the overlying skin via the nipple-areolar orifices, or nipple-oral contact during lactation or sexual contact (Hieken et al., 2016; Rescigno et al., 2001).

Breast milk shares similar bacterial composition with breast tissues and has key roles in infant health (Alpuim Costa et al., 2021; J. Zhang et al., 2021). These two microbiomes share intimate connection and can exert influences across both mother and infant. Specifically, dysbiosis in mother's breast could lead to lactational mastitis (inflammation of the mammary

gland due to an overgrowth of pathogenic bacteria) and consequently impact infant's microbial colonization, immune development, and overall health later in life (Patel et al., 2017; J. Zhang et al., 2021). The fact that lactational mastitis can be treated more efficiently with oral probiotics found in breast milk than with antibiotics further support the hypothesis that gut bacteria could translocate to the breast (Arroyo et al., 2010).

The study of the breast microbiome is still in its infancy, hence only a few studies have examined how the breast microbiome changes with breast cancer. So far, a number of clinical studies have examined the breast microbiota between normal breast and breast cancer, healthy control compared to breast cancer survivors, benign versus malignant disease, nontreated verus chemo-treated breast cancer patients, and between different breast cancer subtypes (Laborda-Illanes et al., 2020; Parida & Sharma, 2019a). It is still unclear if there is a difference in the breast microbiota between normal breast tissue and cancerous breast tissue. Urbaniak et al first found no difference between breast tumor tissue and adjacent normal tissue, except for a higher abundance of tumor-promoting *Escheriachia coli* in the tumors (Urbaniak et al., 2014). Costantini et al also found no difference between the two (Costantini et al., 2018). However, later study by Urbaniak et al showed increased in *Bacillus*, *Enterobacteriaceae*, *Staphylococcus*, *Comamondaceae* and *Bacteroidetes* in breast tumors compared to normal tissue from healthy women. Xuan et al also observed an increase in *Methylobacterium radiotolerans* in ER+ breast tumors and enriched *Sphingomonas yanoikuyae* in healthy adjacent breast tissues (Xuan et al., 2014). Those breast tumors also had lower baseline expression of antibacterial response genes and total bacterial DNA compared to healthy breast tissues, which inversely correlated with advanced disease (Xuan et al., 2014). Wang et al similarly found decreased *Methylobacterium*

and increased *Corynebacterium*, *Staphylococcus*, *Actinomyces* and *Propionibacteriaceae* in patients with invasive breast carcinoma compared to noncancerous tissues from healthy individuals (Wang et al., 2017). Thompson et al also observed an increase in *Mycobacterium* spp. in breast tumors compared to normal adjacent tissue (Thompson et al., 2017).

When healthy volunteers and breast cancer survivors were compared, significant differences were observed in the microbial composition of their nipple fluids (Chan et al., 2016). Women with malignant disease also have significantly different breast microbiota compared to that of women with benign disease (Hieken et al., 2016). Specifically, decreased *Bacteroidaceae* and increased *Agrococcus* were associated with breast cancer malignancy in a cohort of Chinese patients (Meng et al., 2018). Each breast cancer subtype also displays its own unique viral, bacterial, fungal, and parasitic signatures found in the breast tumor microenvironment (Banerjee et al., 2018; Banerjee et al., 2015). ER+ and HER2+ breast tumors were more similar in patterns to each other than triple-negative breast tumors (Laborda-Illanes et al., 2020). The breast microbiota also was altered when patients received neoadjuvant chemotherapy prior to surgery as compared to the initial non-treated tumors or those that developed distant metastases (Chiba et al., 2020). Overall, the breast microbiome has been shown to be diverse and distinctive, and can be altered by the presence of active breast cancer, breast cancer history, malignancy, metastases and chemotherapy. Taken together, these observational clinical data suggest a strong association between the breast microbiome and risk of breast cancer and/or disease progression. However, many more investigations need to be conducted in order to understand the functional significance or causal relationship between the diversity of the microbiome and breast cancer etiology.

Microbiome Dysbiosis and Breast Cancer

Dysbiosis is an abnormal stage or maladaptation of the microbiome due to disturbances. Microbial dysbiosis that is associated with neoplastic diseases is termed oncobiosis (Thomas & Jobin, 2015). Recent development suggests that oncobiosis may play a pathologic role in multiple cancers, including breast cancer (Kovács et al., 2021). Hill et al was the first study to hypothesize that gut bacteria may have pathological roles in breast cancer (Hill et al., 1971). Numerous epidemiological studies in humans and mice both associated antibiotic use with increased breast cancer risk (Friedman et al., 2006; Kirkup et al., 2019; McKee et al., 2021; Simin et al., 2020; Tamim et al., 2008; Velicer et al., 2004; Velicer et al., 2006; Wirtz et al., 2013). In addition, factors known to modulate the microbiome such as consumption of probiotics, prebiotics, and nutrition are associated with decreased breast cancer risk (Guinter et al., 2018; Jiang & Fan, 2021; Maroof et al., 2012; Newman et al., 2019; Ranjbar et al., 2019; Y. Wu et al., 2021). Well-known risk factors for breast cancer including age, high level of circulating estrogen, alcohol consumption, obesity, low level of physical activity, early menarche, high breast density, and periodontal disease have all been associated with changes in the microbiome (Frugé et al., 2020; Jones et al., 2019; Kovács et al., 2021; Parida & Sharma, 2020; Wang et al., 2017; Wu et al., 2020; X. Zhang et al., 2021).

Changes in multiple microbial communities were observed in breast tissues, breast tumors, milk ducts, distal gut and the urinary tract (Chan et al., 2016; Kovács et al., 2021; Smith et al., 2019; Urbaniak et al., 2016; Wang et al., 2017; Wu et al., 2020; Xuan et al., 2014; Zhu et al., 2018). The breast microbiome in particular was altered in the presence of a benign or invasive breast tumor, presence of distant metastases, or treatment with chemotherapy (Laborda-

Illanes et al., 2020). Specific microbial signatures further correlate with breast cancer subtypes as well as clinical outcome (Banerjee et al., 2021). Together, these data suggest that dysbiosis induced by various causes may contribute to breast cancer development. Thus, it is not surprising that the microbiome has now been recognized as a part of the tumor microenvironment, believed to play important roles in immune suppression and/or supporting tumor growth (Rao Malla et al., 2022).

Potential Mechanisms of Microbiome Modulating Breast Cancer

Recent studies have indicated associations between the microbiome (whether in the gut or the breast) and breast cancer risk or disease progression. However, a direct causal relationship between the two has yet to be established. Even more elusive are mechanisms by which the microbiome influences breast cancer development. A more realistic scenario could be that the microbiome and breast cancer coevolve together and thus modulate each other. Nonetheless, there are a number of mechanisms proposed to explain how the microbiome alters breast cancer (Figure 1):

1) Modulation of estrogen metabolism and circulating levels, as estrogen is a critical promoter of breast cancer growth.
2) Modulation of the immune system and inflammation, as the immune system plays important roles in tumor surveillance and chronic inflammation is known to contribute to carcinogenesis.

3) Production of pro/antitumor bacterial metabolites, as numerous metabolites are already known to affect breast cancer cell behavior.

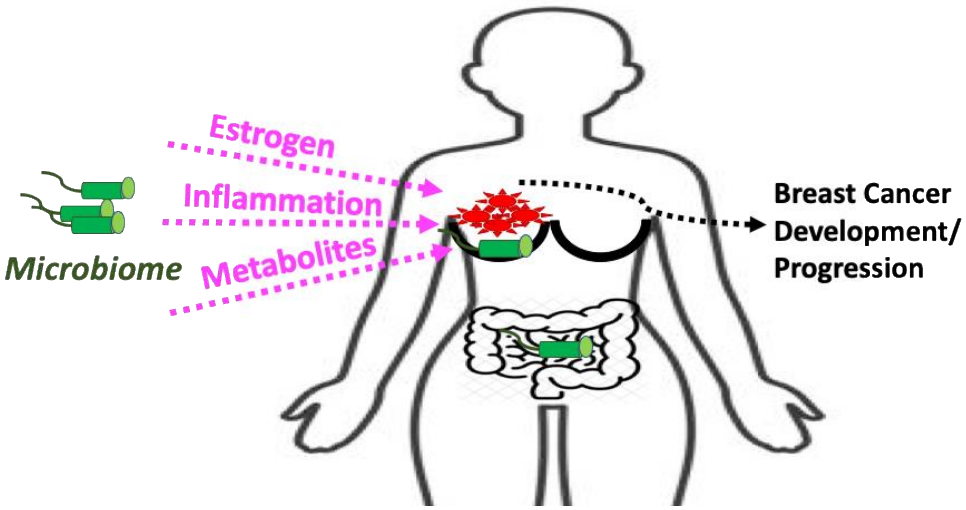


Figure 1. Potential Mechanisms by which the Microbiome Modulates Breast Cancer. The microbiome, whether in the gut or the breast, could modulate estrogen metabolism, enhance inflammation, and produce metabolites to alter breast cancer development.

Microbiome-Cancer Mechanism 1: Modulation of Estrogen Metabolism

Estrogen is one of the most important promoters of breast cancer cell proliferation, and hormonal dysregulation is a major risk factor for breast cancer (Laborda-Illanes et al., 2020; Parida & Sharma, 2019a). Estrogen metabolism in humans is complex and requires participation from multiple enzymes in multiple organs. In premenopausal women, estrogen is produced predominantly by ovaries and functions in an endocrine fashion (Samavat & Kurzer, 2015; Simpson, 2003). In contrast, estrogen is mainly synthesized by extragonadal tissues (including the adrenal glands, adipose tissues of the breast, bones and brain) in postmenopausal women and acts in a paracrine or intracrine manner (Quince et al., 2017). Endogenous estrogen exits in three forms: 17β-estradiol (E2) in premenopausal women, estrone (E1) in postmenopausal women, and estriol (E3) in pregnant women (Parida & Sharma, 2019a). Estrogen metabolism occurs

primarily in the liver, where parental estrogens E1 and E2 become hydroxylated, conjugated, and excreted into the gastrointestinal lumen with bile. In the gut, conjugated estrogen and its metabolites are deconjugated into various estrogen metabolites (free estrogens) by certain gut bacteria. Then, free estrogens are reabsorbed in the distal gut, passed through enterohepatic circulation, and circulated to other tissues including breasts (Yang et al., 2017; J. Zhang et al., 2021).

Thus, bacteria in the gut microbiome play critical roles in modulating estrogen metabolism, reabsorption, excretion, and bioavailability. This group of bacteria, called estrobolome, are capable of producing β-glucuronidase enzymes to metabolize estrogen (Plottel & Blaser, 2011). β-glucuronidase enzymes are encoded by the *gus* (commonly found in *Firmicutes*) and *BG* genes (common in *Bacteroidetes* and *Firmicutes*) (Gloux et al., 2011; Mikó et al., 2019). B-glucuronidase bacteria are predominantly found in *Clostridia* families (*Clostridium leptum* and *Clostridium coccoides*), *Ruminococcaceae* families, and *Escherichia/Shigella* group (Fernández et al., 2018). In addition, bacterial β-glucuronidase can deconjugate environmental pollutant/endocrine disruptors (xenobiotics and/or xenoestrogens), increasing their reabsorption and half-life in the body (Fernández et al., 2018; Yang et al., 2017). Gut bacteria can also metabolize estrogen-like compounds or estrogen mimics from dietary sources, such as phytoestrogens like isoflavones and lignans (Parida & Sharma, 2019a). Given these important functions in estrogen metabolism, perturbations in the gut microbiome (estrobolome) can lead to elevated levels of circulating estrogen, contributing to breast cancer development. Indeed, several studies have demonstrated association between gut microbiota, levels of urinary and circulating estrogen metabolites, and risk of breast cancer (Falk et al., 2013;

Flores et al., 2012; Fuhrman et al., 2014; Fuhrman et al., 2012; Zengul et al., 2021; J. Zhang et al., 2021).

Microbiome-Cancer Mechanism 2: Modulation of the Immune System and Inflammation

It is well established that the immune system plays important roles in cancer initiation and progression. As the microbiome is known to modulate the immune system, it is thought that the microbiome would affect breast cancer by modulating the immune system. Recent studies found that expression of immune-related genes and the presence of the immune infiltrates in the breast tumors were associated with better clinical outcome (Savas et al., 2016). While the presence of cytotoxic CD8+ T cells was associated with better outcome, infiltration of FOXP3+, CD4+ regulatory T cells (Tregs) was correlated with increased stage of breast cancer progression and associated with worse prognosis (Ali et al., 2014; Bates et al., 2006; Gupta et al., 2011; Liu et al., 2014). This suggests that the presence of immunosuppressive Tregs could promote immune evasion and thus tumor progression (Laborda-Illanes et al., 2020). Commensal microbiota and their metabolites (butyrate and propionate) have been shown to modulate/increase colonic Tregs to exert potent anti-inflammatory effects (Furusawa et al., 2013; Laborda-Illanes et al., 2020; Smith et al., 2013). On the other hand, commensal gut bacteria are also known to aid in the maturation of cytotoxic CD8+ T cells, which is important for an anti-tumor response (Gritzapis et al., 2008; Parida & Sharma, 2020).

Mucosal secretory immunoglobulin A (IgA), another component of the immune system, has been associated with breast cancer (Goedert et al., 2018). IgA plays important roles in maintaining the integrity of the mucosal barrier and regulates the intestinal microbiome as it is known to coat certain microbes. (Ashida et al., 2011; Pabst, 2012; Peterson et al., 2007).

Interaction between IgA and the gut microbiome could potentially present another route by which the microbiome impacts breast cancer development.

Since chronic inflammation has been associated with increased breast cancer risk, it could be another mechanism by which the microbiome promotes tumor development (Crusz & Balkwill, 2015). Gut bacteria, through their expression of Patterns Associated with Pathogenic Microorganims (PAMPs) including lipopolysaccharides/LPS, flagellin, lipoteic acid, peptigoglycans, can activate Toll-Like Receptors (TLRs) on innate immune cells to stimulate inflammation (Pandey et al., 2015). Chronic activation of TLRs leads to the release of proinflammatory cytokines including IL-6 and TNF-α in the tumor microenvironment, which promote tumor proliferation and invasion (Mantovani et al., 2008; Pandey et al., 2015). Breast cancer cells, in turn were found to express varying levels of different TLRs (Bhatelia et al., 2014). In addition, the microbiome can regulate levels of inflammatory neutrophils and leukocytes (Parida & Sharma, 2020). Elevated numbers of neutrophils is associated with worse survival and higher rates of recurrence for breast cancer patients (Azab et al., 2012; Margolis et al., 2007). In mice, neutrophils are required for the development of mammary tumors induced by gastric administration of *Helicobacter hapticus* (Lakritz et al., 2015). Moreover, *H. hapticus* enteric infection was found to induce breast cancer via a TNF-α-dependent innate immune response (Rao et al., 2006). Thus, recruitment and activation of neutrophils\leukocytes could be another way by which the microbiome promotes tumor progression. These data also suggest that low-grade enteric infection early in life may induce a tolerance toward tumorigenesis (Parida & Sharma, 2020). Taken together, the modulation of the immune system and local or systemic

inflammation by the microbiome could be critical mechanisms for breast cancer development and progression.

Microbiome-Cancer Mechanism 3: Production of Pro-/Anti-tumor Bacterial Metabolites

Although the host can regulate its microbiome through its immune system or lifestyle behaviors, microbes are also known to modulate the host through secretion of a wide range of bioactive metabolites that act like hormones (Mikó et al., 2019). In addition to circulating metabolites from the distal gut microbiome, the breast microbiome is also capable of producing signaling molecules to influence the local breast or tumor microenvironment (Parida & Sharma, 2020). These metabolites can act through various pathways to modulate gene expression or signal transduction in the host (J. Zhang et al., 2021). In fact, a number of bacterial metabolites were shown to modulate breast cancer growth, either promoting and/or inhibiting it depending on the molecule: short chain fatty acids (SCFAs), secondary bile lithocholic acid (LCA), cadaverine, indole derivatives, and bacteriotoxin.

Short chain fatty acids (SCFAs) are the most well-studied microbial metabolites. They are produced from dietary polysaccharides in the small intestine, with acetate and propionate being made by *Bacteroides* and butyrate by *Firmicutes (Mikó et al., 2019)*. Human serum concentration of SCFAs ranges between 10-100μM (Clausen et al., 1991; Ktsoyan et al., 2016). SCFAs can activate various GPCRs or orphan free fatty acid receptors (FFARs). SCFAs are required for the induction of Tregs and various immune functions, which are important in tumor progression (Arpaia et al., 2013). In breast cancer cells, SCFAs have been shown to modulate various cancer hallmarks, including cell proliferation, apoptosis, invasion, and metabolism (Mikó et al., 2019). They can be used as an energy source by breast cancer cells, stimulating oxidative

phosphorylation and oxygen consumption (Rodrigues et al., 2015). In addition, SCFAs can inhibit histone deacetylases to modulate epigenetic processes, which are often involved in the inactivation of tumor-suppressor genes in breast cancer (Jeffery & O'Toole, 2013; Laborda-Illanes et al., 2020). Butyrate has been shown to activate expression of genes, previously silenced in cancer cells including p21 and BAK (Berni Canani et al., 2012). SCFAs can act directly on breast cancer cells, or indirectly through immune modulation. It may have positive or negative effects on breast cancer, depending on the context (Mikó et al., 2019).

Lithocholic acid (LCA) is a secondary bile acid made by anaerobic bacteria in the small intestine (mainly *Clostridiales)* (Ridlon et al., 2006). Bacteria in this group use the enzyme 7α/β-hydroxysteroid dehydroxylase to produce LCA from primary bile acids (Ridlon et al., 2016). The bile acids found in the breast all originate from the gut (Javitt et al., 1994). Here, LCA is found to inhibit breast cancer proliferation and invasion (Mikó et al., 2018). At physiological concentrations (30–50nM in serum or <1μM in breast tissue), LCA signals through the Takeda G-protein coupled receptor (TGR5) to inhibit proliferation, epithelial-to-mesenchymal transition (EMT), VEGF production, and metastasis (Mikó et al., 2018). LCA also induces antitumor immunity and regulates oxidative phosphorylation at this concentration (Lefebvre et al., 2009; Mikó et al., 2018). At supraphysiological concentrations (>1 μM), LCA acts through the Farnesoid X Receptor (FRX) to inhibit fatty acid biosynthesis, and induce multidrug resistance proteins and cell death (Luu et al., 2018; Swales et al., 2006). LCA is associated with antineoplastic effects. Compared to healthy controls, early stage breast cancer patients had reduced serum LCA levels and reduced key enzymes used in LCA synthesis (Mikó et al., 2018).

Serum LCA also negatively correlates with the proliferation marker, Ki67 in breast cancer (Tang et al., 2014).

Cadaverine is produced from lysine degradation, predominantly by bacteria expressing cadaverine synthesis enzymes including *Shigella flexneri*, *Shigella sonnei*, *Escherichia coli*, and *Streptococci* (de las Rivas et al., 2006; Kovács et al., 2019; Mikó et al., 2019). Cadaverine functions by activating trace amine-associated receptors (TAAR1,2,3,5,8,9) (Vattai et al., 2017). Cadaverine exerts antineoplastic effects on breast cancer. Hence, the capacity to produce cadaverine is reduced in early stage breast cancer (Kovács et al., 2019). At physiological serum concentration (100–800nM), cadaverine inhibited breast cancer cell proliferation, EMT, migration, invasion and metastasis, while having no effect on untransformed breast cells (Kovács et al., 2019; Löser et al., 1990). Furthermore, cadaverine altered the cell metabolism to be more glycolytic and reduced the proportion ALDH1+ cancer stem cells in 4T1 murine cell line (Kovács et al., 2019).

Indole derivatives are metabolized from dietary tryptophan by *Lactobacilli* (Parida & Sharma, 2020). Indoles activate the Aryl hydrocarbon receptor (AhR) pathway, which is important in immune regulation (Postler & Ghosh, 2017). Furthermore, tryptophan metabolism and high AhR signaling were associated with risk of breast cancer (Bekki et al., 2015; Cao et al., 2015; Dewi et al., 2017; Puccetti et al., 2015). For example, amplified tryptophan metabolism and upregulated AhR signaling prevent apoptosis of cancer cells (Bekki et al., 2015; Cao et al., 2015). Bacterial indoles are also known to interact with the pregnane X receptor (PXR) and modulate mucosal integrity via its crosstalk with TLR4 (Venkatesh et al., 2014). Activation of

PXR was shown to inhibit proliferation and induce apoptosis of breast cancer cells (Parida & Sharma, 2020; Pondugula et al., 2016).

Bacteriotoxins, or toxins produced by bacteria, are another class of compounds produced by commensal bacterial (Parida & Sharma, 2020). Metagenome data indicate that approximately 4875 different bacteriotoxins are encoded by bacteria across the human body (Zheng et al., 2015). Since bacteriotoxins are made to inhibit the growth of other pathogenic bacteria, these bacteriocins could potentially be used to inhibit pathogen colonization to improve cancer outcome (Parida & Sharma, 2020). However, a previous study showed that colibactin, produced by several *Escherichia coli* and *Staphylococcus epidermidis* isolates cultured from normal breast tissue of breast cancer patients, was able to induce DNA double-stranded breaks and genomic instability in HeLa cells (Urbaniak et al., 2016). Another bacteriotoxin, *B. fragilis* toxin secreted by the enterotoxigenic *Bacteoroides fragilis* found in both the gut and the breast microbiome, was shown to induce murine breast tumor growth and metastatic progression through activation of the β-catenin and Notch1 pathway (Parida et al., 2021). These suggest that certain bacterial toxins could have direct tumorigenic effects on cancer cells (Fernández et al., 2018). Other well-known bacterial toxins such as LPS and Lysophosphotidic acid (LPA) are associated with breast cancer through their interaction with respective TLR/LPAR signaling (Kovács et al., 2021). Many bacteriotoxins are yet to be discovered and characterized for their impacts on breast cancer. Thus, this presents an exciting opportunity for identification of novel targets to improve therapeutic strategies for breast cancer.

Interactions Between the Microbiome and Breast Cancer Therapies

It became evident that the microbiome plays an important role in the host's response to cancer therapies, including chemotherapy, radiation, targeted and immunotherapy. Intestinal bacteria are known to biotransform a number of anti-cancer drugs, activating or inactivating them via enzymatic reactions or physical interactions (Haiser & Turnbaugh, 2013; Mikó et al., 2019; Parida & Sharma, 2019b). The microbiome can also modulate the host's immune response needed for therapeutic efficacy of certain drugs (Manepalli et al., 2013; Viaud et al., 2013). Thus, the presence or absence of the microbiome or even particular bacterial species can affect drug absorption, bioavailability, efficacy and toxicity (Mikó et al., 2019; Roy & Trinchieri, 2017). It is important to note that the microbiome-drug interaction is bidirectional, in the sense that the microbiome can modulate the drug response while the drug itself can also alter the composition and function of the microbiome (Mikó et al., 2019). Ultimately, the goal of cancer therapy is to maximize efficacy while minimizing side effects. The microbiome, also called our forgotten organ, offers a new opportunity to improve treatment and possible outcomes for patients with breast cancer.

Chemotherapy and the Microbiome

A number of chemotherapies used in treatment of breast cancer are modulated by the intestinal microbiota (Mikó et al., 2019; Viaud et al., 2015). Specifically, the DNA-alkylating agent cyclophosphamide (CTX) was shown to mobilize certain gram-positive bacteria in the gut to secondary lymphoid organs to generate Th17 and Th1 cells necessary for long term antitumor immune response (Viaud et al., 2013). Commensal bacteria *Enterococcus hirae* and *Barnesiella intestinihominis* were able to restore the antitumor response of CTX in germ-free mice (Daillère

et al., 2016). *Lactobacillus* strains, on the other hand, are protective against CTX-induced immunosuppression and mucosal injury (Salva et al., 2014; Xie et al., 2016; Yang et al., 2013). Anthracyclines, such as doxorubicin, modulates composition and mobility of the microbiome, while being metabolized by several strains of *Streptomyces* (Alexander et al., 2017; Cox et al., 2014; Parajuli et al., 2018; Westman et al., 2012). Other therapies including 5-fluoracil (5FU) and gemcitabine are metabolized by the microbiome to produce either inactive or active compounds (Alexander et al., 2017; Mikó et al., 2019). Both of these agents are also bactericidal and modulate the composition of the microbiome (Mikó et al., 2019). Probiotic usage was shown to protect from 5FU-induced mucositis and dysbiosis (Hamouda et al., 2017; H.-L. Li et al., 2017; Yeung et al., 2015). Lastly, platinum compounds such as oxaliplatin and cisplatin lost their anti-tumor efficacy in the absence of the microbiome (Iida et al., 2013; Parida & Sharma, 2019a). Administration of the probiotic *Lactobacillus acidophilus* restored the anti-tumor response of cisplatin in antibiotic-treated mice (Gui et al., 2015). Overall, certain chemotherapies depend on the microbiome to become activated and to modulate the immune system in order to exert their desired anti-tumor effect. These drugs can also cause dysbiosis and toxicities, which can be ameliorated with the administration of certain probiotic bacteria.

Radiotherapy and the Microbiome

Although some studies suggest a strong association between the presence of the microbiome and the efficacy of radiotherapy, the relationship is not well understood. Some of the radiation-associated toxicities can be attributed to intestinal dysbiosis and the subsequent altered immune response (Parida & Sharma, 2019a). Thus, a number of probiotic preparations

containing *Lactobacillus* have been shown to protect against gut-associated toxicities induced by radiation (Blanarova et al., 2009; Jones et al., 2015; Jones et al., 2013; Sharma et al., 2012).

Targeted/Immunotherapy and the Microbiome

Therapies targeting estrogen signaling or resulting in DNA damage in breast cancer are influenced by the microbiome. Certain bacteria are able to metabolize the DNA damaging agents, taxanes, and taxanes in turn can interfere with the composition of the microbiome as well as the bacterial LPS-stimulated immune response (Byrd Cynthia et al., 1999; Dou et al., 2015; Oelschlaeger & Tall, 1997; Zhou et al., 2013). Although metabolism of SERMs (Tamoxifen and Raloxifene) is not regulated by bacteria, SERMs are known to modulate the composition of the microbiome by being bactericidal toward certain bacterial species (Laborda-Illanes et al., 2020; Mikó et al., 2019).

Other DNA damaging agents such as PARP inhibitors also modulate the composition of the gut microbiome (Larmonier et al., 2016; Vida et al., 2018). As for targeted therapies, Mikó et al noted that there was no published interaction between estrogen disruptors (aromatase inhibitors, navelbine, GNRH-analogs) and the microbiome (Mikó et al., 2019). Immune checkpoint inhibitors including anti-CTLA-4 and anti-PD-L1 antibodies used to stimulate the anti-tumor immune response required the presence of the microbiome (Alexander et al., 2017). Specifically, *Bifidobacterium* spp. facilitates the anti-tumor immune response induced by anti-PD-L1 (Sivan et al., 2015). *Bacteroidales fragilis*, on other hand, is able to reduce colitis induced by anti-CTLA4 (Vétizou et al., 2015). However, no interaction was reported thus far between the targeted antibodies used to treat breast cancer with the microbiome (Mikó et al., 2019).

Bugs as Drugs: Harvesting the Power of the Microbiome for Therapeutic Purposes

As studies started to unveil the importance of the microbiome in health and disease, particularly with respect to cancer initiation, progression and response to therapies, it is only natural to find ways to manipulate the microbiome for our benefits. A number of strategies including diet, probiotics, fecal transplant and engineered bacteria are being investigated as novel cancer treatment. The ultimate goal is to restore the host's microbiome to a healthy state in order to minimize disease progression and drug toxicities, while maximizing drug efficacy. Some of these novel microbiome-based therapies have already reached the bedside of patients and showed promising results.

Diet and Probiotic/Prebiotic/Postbiotics Supplement as Cancer Therapy

Diet is one of the most convenient and effective method to alter the gut microbiome (Carmody et al., 2015; Parida & Sharma, 2021). Studies have examined either general diet trends or the use of probiotic, prebiotic, or postbiotics as dietary supplements. The Mediterranean diet, which includes significant plant polyphenols and wine consumption, has been associated with lower incidence of breast cancer (Buckland et al., 2013; Fung et al., 2006; Grosso et al., 2013; Murtaugh et al., 2008; Trichopoulou et al., 2010). Furthermore, consumption of this diet in non-human primates has been shown to modulate the gut microbiome, enriching for L*actobacillus, Clostridium, Faecalibacterium,* and *Oscillospira* compared to the Western diet (Nagpal et al., 2018). The Mediterranean diet also drastically increased the abundance of protective *Lactobacillus* in the breast microbiome (Shively et al., 2018). In contrast, switching to a high-fat, high-sugar Western diet shifted the metabolic and gene expression profiles of the gut microbiome within a day, leading to reduced bacterial diversity as well as decreased health-promoting *Bacteroides*

population (Turnbaugh et al., 2009). Thus, several clinical studies are investigating effects of diet interventions upon breast cancer outcomes (Kirkham et al., 2021; Lugtenberg et al., 2021; Pierce et al., 2007; Zuniga et al., 2019).

Probiotics, or the use of living microorganisms to promote health, is another venue of active research (Ranjbar et al., 2019). Probiotics may be consumed as fermented food or those containing live cultures such as yogurt and kefir. Some of the most common probiotic bacterial genus include *Lactobacillus* and *Bifidobacteria*, *Streptococcus, Enterococcus, Escherichia,* and *Bacillus* (Fijan, 2014). Recent studies demonstrated beneficial effects of several probiotics in the prevention and treatment of breast cancer. *In vivo* and *in vitro* studies showed that probiotic treatment (mainly *Lactobacillus* and *Bifidobacteria* strains) reduced breast tumor growth, angiogenesis, metastasis and inflammation (Ranjbar et al., 2019). In clinical studies, consumption of *Lactobacillus casei Shirota*, or yogurt and fermented milk products displayed an inverse correlation with breast cancer incidence (Lê et al., 1986; Toi et al., 2013; van't Veer et al., 1989). Furthermore, probiotic administration to breast cancer survivors positively influenced the microbiome and metabolic profiles (Pellegrini et al., 2020). Probiotic supplements significantly reduced the incidence of chemotherapy-related cognitive impairment and alleviated gastrointestinal disturbances induced by chemotherapy or radiation in breast cancer patients (Abd El-Atti et al., 2009; Juan et al., 2022). Another promising route of research is the use of probiotic bacteria such as *Akkermansia muciniphila* to improve patient's response to anti-PD-1 immunotherapy (Gopalakrishnan et al., 2018; Routy et al., 2018). More studies are needed to delineate the specific patient population who would benefit from specific strains of probiotics.

Another strategy is to administer prebiotics (dietary components/fibers that stimulate the growth of probiotic bacteria in the gut) or synbiotics (combining probiotics and prebiotics). Clinical studies showed that consumption of synbiotics by overweight postmenopausal women with breast cancer led to improvements in term of inflammatory makers, oxidative markers, BMI and overall quality of life (Navaei et al., 2020; Raji Lahiji et al., 2021; Saneei Totmaj et al., 2022; Vafa et al., 2020). Synbiotics may best reduce side effects associated with chemotherapy when given during chemotherapy, as seen in patients with esophageal cancer (Motoori et al., 2017).

A relatively newer development on the block is postbiotics (Vrzáčková et al., 2021). Postbiotics refers to the use of microbial metabolites as food supplements to promote health (Tsilingiri & Rescigno, 2013). They may include bacterial lysates and supernatant, components of cell walls, enzymes, exopolysaccharides, short chain fatty acids, and other metabolites produced by bacteria (Żółkiewicz et al., 2020). The use of postbiotics is based on the observation that the beneficial effects of the microbiome are mediated, at least partly, by the secretion of metabolites (Żółkiewicz et al., 2020). Postbiotics are known to exert various health-promoting effects upon consumption, including anti-microbial, anti-inflammatory, anti-oxidant, and anti-cancer (Delgado et al., 2020; Homayouni Rad et al., 2020; Homayouni Rad et al., 2021). As an adjuvant for cancer therapy, the anti-cancer effects of postbiotics are strongly associated with their modulation of the immune system (Homayouni Rad et al., 2021; Vrzáčková et al., 2021). Postbiotics are generally considered a safer alternative than live bacteria in probiotics (Rad et al., 2020). It is a field of growing interest with potentials for novel therapeutic development, needing more research.

Fecal Microbiota Transplantation as Cancer Therapy

The most innovative, drastic and successful application of the microbiome in the last decade is arguably Fecal Microbiota Transplant (FMT) (Chen et al., 2019). FMT is defined as the direct transfer of gut microbiota from a healthy donor to a sick recipient with the goal of restoring the intestinal microbiota to its healthy state (Wang et al., 2019). Fresh or frozen feces may be processed and delivered to the patient via oral capsule, nasogastric tube, nasoduodenal tube, enema, or colonoscopy (Borody et al., 2019; Cammarota et al., 2014). Feces transplant was first reported as a "yellow soup" being used to treat food poisoning or severe diarrhea 1700 years ago in China (Zhang et al., 2012). The practice was seldomly used until the first documented case of recurring *Clostridium difficile* enterocolitis being cured by FMT in 1983 (Schwan et al., 1983). Since 2013, FMT has been approved for treating recurring or refractory *C. difficile* infection with up to 90% efficacy (Konturek et al., 2015). FMT is generally considered safe with minor adverse side effects (Brandt et al., 2012; Wang et al., 2019). However, there are concerns with accidental transmission of pathogens, and long-term outcomes that are yet to be observed, such as induction of chronic diseases due to altered microbiota or immunomodulation, or transmission of unrecognized infectious agents that cause illness years later (Wang et al., 2019). Thus, researchers are considering safer alternatives to whole fecal transplant, such as using "lab-grown microbiota consortium;" however, their efficacy compared to FMT is yet to be fully investigated (Parida & Sharma, 2021).

Recent studies have started to expand FMT to other intestinal diseases, including inflammatory bowel diseases and intractable constipation, as well as extra-intestinal diseases such as metabolic disease, neuropsychiatric disorders, allergic and autoimmune diseases (Xu et

al., 2015). As roles for the microbiome in cancer are being recognized, FMT gains attention as a potential therapy for various type of cancers and cancer-associated complications (Chen et al., 2019). Initial studies reported promising results using FMT to treat colon, liver, and pancreatic cancers (Chen et al., 2019). FMT also proved important in reducing chemotherapy-induced toxicities and having proper response to immunotherapy (Chang et al., 2020; Gopalakrishnan et al., 2018; Parida & Sharma, 2021; Routy et al., 2018). However, to date, the application of FMT in breast cancer has yet to be shown in published studies, and remains to be explored.

Attenuated and Engineered Bacteria as Cancer Therapy

Dr. William Coley was recognized as the first to use bacteria against cancer, by inoculating more than 1000 cancer patients with a mixture containing heat-inactivated *Streptococcus pyogenes* and *Serratia marcescens* to induce tumor regression (Coley, 1991). With better understanding of the host-microbe-tumor interaction and development of recombinant DNA technology, researchers are returning to this old strategy with new perspectives and novel tools (Zhou et al., 2018). Several bacterial species (*Clostridia, Lactococcus, Bifidobacteria, Shigella, Vibrio, Listeria, Escherichia*, and *Salmonella*) have been found to naturally colonize and proliferate within solid tumors, which often results in tumor shrinkage and clearance (Song et al., 2018). These bacteria are often obligate or facultative anaerobic bacteria that selectively colonize the hypoxic and necrotic regions in the tumors, despite systemic administration (Kasinskas & Forbes, 2007; Zhou et al., 2018). One well-known, efficient anti-tumor bacterial strain is the attenuated auxotrophic *Salmonella typhimurium* mutant, which has been shown to selectively infect, attack, and cure breast tumors in nude mice (Zhao et al., 2006). These bacteria generally exert their anti-tumor effects by competing with tumor cells for oxygen and available

nutrients, resulting in the death of tumor cells (Danino et al., 2013; Yaghoubi et al., 2019). In addition, these bacteria may inhibit tumor growth by activating the anti-tumor immune response or releasing anti-tumor substances (toxins, peptides, enzymes) (Song et al., 2018). Genetic engineering of bacteria can further enhance their safety, tumor colonization, selectivity and toxicity against cancer cells (Zhou et al., 2018). Bacteria can also be made to selectively target tumors using controllable drug/gene delivery vehicles (Fujimori, 2006; Li et al., 2022). Potential agents that can be delivered to tumors are cytokines, cytotoxic agents, immunomodulators, prodrug-converting enzymes, and/or siRNAs (Duong et al., 2019). Strains of *Bifidobacterium, Clostridium*, and *Salmonella* have been shown to be safe and effective for this purpose in various cancer models (Fujimori, 2006).

Although bacterial therapy showed exceptional results in preclinical models, only a few of these therapeutic, live tumor-targeting bacterial strains have actually advanced to clinical trials (Zhou et al., 2018). Translation of replication-competent bacteria is challenging, due to regulations and concerns regarding infection-associated toxicities (Zhou et al., 2018). Thus far, a handful of pilot clinical trials using oncolytic *Clostridium* and attenuated *Salmonella* strains have shown tumor colonization and lysis with few adverse effects (Carey et al., 1967; Heppner & Möse, 1978; Nemunaitis et al., 2003; Roberts et al., 2014; Toso et al., 2002; Zhou et al., 2018). Despite these hurdles, bacterial therapy carries many advantages worth exploring, and holds the potential to become a powerful weapon in our fight against cancer.

Microbiome in Breast Cancer: Future Directions and Challenges

While dysbiosis is a maladaptive stage of the microbiome, eubiosis is defined as a balanced microbiome in which a diverse microbial community is living in harmony (Parida &

Sharma, 2021). A balanced microbiome, as part of our "supra-organism," is essential to health and wellbeing. Recent studies demonstrated an association between breast cancer and dysbiosis within the local breast environment and\or the distal intestinal microbiome (Kovács et al., 2021). In fact, many of the well-known risk factors for breast cancer are closely linked to an altered microbiome (Frugé et al., 2020; Jones et al., 2019; Kovács et al., 2021; Parida & Sharma, 2020; Wang et al., 2017; Wu et al., 2020; J. Zhang et al., 2021). Studies have found the breast microbiome to be significantly different between breast tumor compared to normal breast tissue, and between healthy control and breast cancer patients (Laborda-Illanes et al., 2020; Parida & Sharma, 2019a). Different breast subtypes also have their own unique microbial signatures that correlate with clinical outcome (Banerjee et al., 2018; Banerjee et al., 2015). Moreover, the presence or absence of particular microbes can significantly impact responses to breast cancer therapies and associated toxicities (Mikó et al., 2019; Roy & Trinchieri, 2017). Altogether, these observations suggest that patient's microbial signatures may be predictive of disease severity, clinical outcome, and treatment response (Parida & Sharma, 2021). As breast cancer is known to be a heterogenous disease, it would be novel and advantageous to make use of the microbiome as another biological marker to optimize patient's prognosis and cancer treatment. Since the microbiome is so diverse and very malleable by many elements, the current challenge is to identify an appropriate microbial signature that is effective and applicable across patients. Overall, the microbiome is starting to shine as the next milestone in the era of personalized medicine.

Many mysteries are yet to be revealed in the relationship between the microbiome and breast cancer. Both the gut and the breast microbiome seem to play important roles in breast

cancer initiation, progression, metastasis, as well as response to therapy. However, to make effective use of the microbiome in the context of breast cancer treatment, we first must understand the mechanism by which breast cancer is influenced by the microbiome. The first challenge with a microbiome study is that it is overwhelming in term of number of microbes present, which makes it very challenging to identify which microbes cause cancer and which are beneficial. Studies have identified a number of alpha-bugs, namely those that are known to drive tumorigenesis, but many are yet to be discovered (Parida et al., 2021). The second challenge is that mechanisms by which microbes exert their effects on cancer cells are poorly understood. It is clear that microbes have systemic effects on distal sites as well as local effects on the tumor microenvironment. They can also have pleiotropic effects on various systems that together contribute to cancer inhibition or promotion. Various modes of action have been proposed for breast cancer, including modulation of estrogen metabolism, immunomodulation, and metabolite secretion. Considering the vast number of microbes present and the heterogeneity of breast cancer, it is no doubt that numerous mechanisms by numerous actors are at play. The field is so new that we have barely scratched the surface. As we expand our understanding of how the microbiome influences breast cancer, we can hopefully utilize the knowledge to develop better prevention and treatment strategies for women with breast cancer.

The Gut Commensal Bacterium *Bacillus subtilis* and its Exopolysaccharide

Bacillus subtilis Exposure and Usage

B. subtilis is a Gram-positive bacterium ubiquitously found in the natural environment, from soil, water, tree roots, seaweed to larva gut (Hong et al., 2009; Jeżewska-Frąckowiak et al., 2018). However, in the modern world where clean/urbanized environment and processed foods

are common, exposure to *B. subtilis* is considered as probiotics from unconventional sources (Jeżewska-Frąckowiak et al., 2018). *B. subtilis* is commonly used to ferment a variety of non-dairy, traditional foods in many parts of Asia, such as fermented soybeans called Natto\Miso in Japan or Cheongukjang in Korea, or fermented cabbage called Kimchi in Korea (Jeżewska-Frąckowiak et al., 2018; Kim et al., 2021). *B. subtilis* is also considered a gut commensal bacterium, as it has been isolated from the ileum and feces of healthy humans (Fakhry et al., 2008; Hong et al., 2009). These *B. subtilis* strains found in the gastrointestinal tract (GIT) have adapted to survive there, having the ability to form a biofilm, to anaerobically sporulate and secrete antimicrobials (Fakhry et al., 2008; Hong et al., 2009). As a spore-forming bacterium, *B. subtilis* can survive harsh environments, from extreme heat, pH, salt, dehydration and poor nutrition (Jezewska-Frackowiak et al., 2017). The spores were shown to withstand extreme conditions and long storage (Hoa et al., 2000). Upon oral ingestion, *B. subtilis* spores generally germinate in the upper GIT, proliferate, then resporulate in the small intestine to be excreted in feces (Bernardeau et al., 2017; Hoa et al., 2000). Additionally, animal studies showed that *B. subtilis* can persist in the gut for up to 20 days after its withdrawal from the diet (Bernardeau et al., 2017).

Probiotic *B. subtilis* strains are commonly used as single or mixed type commercial probiotic preparations. Various studies have found *B. subtilis* to have protective antimicrobial, antiviral, and anticancer effects (Lee et al., 2019). To date, *B. subtilis* has not been associated with any adverse outcomes in immunocompetent persons, except for some reports of its association with opportunistic infection in immunocompromised patients (Farrar, 1963; Hoa et al., 2000; Ihde & Armstrong, 1973; Reller, 1973). Not only is it used as a probiotic in humans, *B.*

subtilis is also widely used as a probiotic feed additive to improve animal production (Lv et al., 2020). In addition, *B. subtilis* has been studied extensively due to the ease of genetic manipulation, and used as a model bacterium for studies on physiology and metabolism. *B. subtilis* is also employed as a cell factory for microbial production of chemicals, enzymes, and antimicrobials for use in industry, agriculture, and medicine (Su et al., 2020). One of the latest applications explored *B. subtilis* as a vaccine expression vector for the treatment and prevention of various infectious diseases (Lv et al., 2020).

Bacillus subtilis Exopolysaccharide: Structure

B. subtilis is known to secrete a variety of bioactive molecules, including antimicrobial peptides, polyketides, and bacteriocins (surfactin, bacilysis, and subtilin) (Caulier et al., 2019). *B. subtilis* can also form robust biofilms, which are an assembly of tightly associated bacteria encapsulated in a self-produced extracellular matrix (Vlamakis et al., 2013). Exopolysaccharide (EPS) is an important component in biofilm formation. EPS is thought to provide structural support to the extracellular matrix, and can be secreted into the extracellular matrix or remain bound to the cell surface (Marvasi et al., 2010). EPS is synthesized by the enzymes encoded within the 15 genes *epsA-O* operon. However, it is poorly understood how the operon operates to synthesize EPS or what the structure of EPS entails (Vlamakis et al., 2013). In fact, very few studies are published on the isolation and identification of EPS from *B. subtilis*.

The Knight laboratory is one of the very first to purify and study EPS from *B. subtilis* in the context of inflammatory diseases. The group reported that they isolated EPS from *B. subtilis* DS991 (*sin*R*tas*A mutant) strain, which overproduced and secreted large amounts of EPS into the supernatant (Jones et al., 2014). Specifically, EPS was collected from stationary supernatants

and digested with DNase, RNase, and proteinase K. Then, EPS was precipitated with ethanol, purified by gel filtration on an S1000 column, and finally desalted by dialysis. Jones et al observed very little contamination of proteins or nuclear acids in EPS preparation. On western blots, EPS appeared as a single band at approximately 300 kDa, suggesting that EPS may be one large structure (Jones et al., 2014). Detailed composition and structure analysis of EPS is currently underway.

Bacillus subtilis Exopolysaccharide: Immunomodulation and Mechanism

B. subtilis EPS was shown to have strong immunomodulatory properties. Systemic administration of EPS was found to be protective against a number of T-cell mediated inflammatory disease, including *Citrobacter rodentium* induced acute colitis, systemic *Staphylococcus aureus* infection, house dust mite (HDM)-induced allergic eosinophilia, and acute Graft-versus-Host Disease. The main findings of immunodulation by EPS across various disease models are summarized in Table 1. *C. rodentium* induced colitis is a murine model of human traveler's diarrhea caused by enterohemorrhagic and enteropathogenic *Escherichia coli*. The study was first started when Jones et al found that a single dose of *B. subtilis* spores given orally a day prior to infection was sufficient to protect mice from acute colitis induced by oral gavage with the pathogenic *C. rodentium* (Jones & Knight, 2012). However, spores from *B. subtilis* epsH mutants lacking EPS did not protect from the disease, suggesting that EPS may be the active molecule required for protection (Jones & Knight, 2012). Subsequently, a single injection of *B. subtilis* EPS (100µg given via i.p) was sufficient to prevent the *C. rodentium*-associated intestinal disease (Jones et al., 2014). Mechanistically, EPS was found to induce anti-inflammatory peritoneal macrophages, as adoptive transfer of peritoneal macrophages from EPS-

treated mice conferred the same protection to recipient mice *(Jones et al., 2014)*. These peritoneal macrophages induced by EPS were identified as M2 macrophages that could inhibit both CD4+ and CD8+ T cell activation. Inhibition of CD4+ T cell activity was dependent on TGF-β, whereas $CD8^+$ T cell inhibition required both TGF-β and PD-L1 (Paynich et al., 2017).

Using a mouse model of HDM-induced eosinophilic inflammation, Swartzendruber et al tested whether *B. subtilis* spores or its EPS could prevent the development of allergic disease (Swartzendruber et al., 2019). The study reported that while wild-type *B subtilis* spores could significantly reduce eosinophilia induced by HDM, mutant spores lacking EPS were unable to protect. Moreover, bone marrow derived dendritic cells (BMDCs) pretreated with EPS *in vitro* could also prevent eosinophilia in HDM-treated mice, suggesting that EPS acts on myeloid cells to mediate its protection (Swartzendruber et al., 2019).

In a mouse model of systemic *S. aureus* infection, treatment with *B. subtilis* EPS enhanced survival while reducing weight loss, systemic inflammation and bacterial burden (Paik et al., 2019). Here, EPS was able to reduce bacterial burden and limit T cell activation and inflammation in mice infected with *S. aureus*. In particular, EPS induced duel-functioning macrophages that limit inflammation while having enhanced ROS-mediated capacities to restrict bacterial growth. These macrophages were also shown to limit T cell activation by *S. aureus* superantigens (Paik et al., 2019). EPS was also observed to inhibit the production of gamma interferon (IFN-γ) by NK cells following *S. aureus* infection (Paik et al., 2020).

Table 1. Summary of EPS Immunomodulation in Various Disease Models

Species Model	Disease Model	Effects of EPS/ B subtilis spores	Mechanisms	References
Mouse	*Citrobacter rodentium* induced acute colitis	A single dose of *B. subtilis* spores or EPS i.p injection given a day prior to infection protects mice from acute colitis induced by *C. rodentium*	- EPS induces anti-inflammatory peritoneal macrophages which inhibit T-cell activation through TGF-β and PD-L1 - Requires TLR4	(Jones & Knight, 2012; Jones et al., 2014; Paynich et al., 2017)
Mouse	Systemic *Staphylococcus aureus* infection	EPS limits systemic inflammation, bacterial burden, weight loss, and enhances overall survival	- EPS induces duel-functioning macrophages that limit inflammation, restrict bacterial growth with enhanced ROS, and limit T cell activation by *S. aureus* superantigens - Inhibits the production of IFNγ by NK cells - Requires TLR4	(Paik et al., 2019, 2020)
Mouse	House dust mite (HDM)-induced allergic eosinophilia	*B. subtilis* spores reduce eosinophilia induced by HDM	- EPS acts on myeloid cells to mediate its protection	(Swartzendruber et al., 2019)
Mouse	Acute Graft-versus-Host Disease	EPS ameliorates acute graft-versus-host disease and improved the survival of mice	- EPS-induces inhibitory dendritic cells which inhibit T cell activation in an IDO dependent manner - Requires TLR4	(Kalinina et al., 2021)

The latest findings reported that treatment with *B. subtilis* EPS also ameliorated acute graft-versus-host disease (aGvHD) in mice (Kalinina et al., 2021). In fact, EPS treatment (100µg given via i.p) significantly improved the survival of mice following aGvHD induction. EPS was found to prevent the activation of alloreactive donor T cells, hence limited inflammation and death. Further *in vitro* studies found EPS to have no direct effect on T cells, but indirectly induced inhibitory dendritic cells from BMDCs. These EPS-induced inhibitory dendritic cells inhibited T cell activation in an IDO dependent manner (Kalinina et al., 2021).

Across multiple models of infection and inflammation, *B. subtilis* EPS is able to limit T-cell driven inflammatory diseases. However, EPS does not act directly on T cells themselves (Kalinina et al., 2021). Instead, EPS induces myeloid cells, such as dendritic cells and macrophages in certain compartments, to inhibit T cells (Jones et al., 2014; Kalinina et al., 2021; Paik et al., 2019; Swartzendruber et al., 2019). These suppressive/tolerogenic myeloid cells mediate their effects partly through inhibitory molecules such as TGF-β, PD-L1, and IDO (Kalinina et al., 2021; Paynich et al., 2017).

One important mechanism used by EPS is the requirement for TLR4, along with the adaptor MyD88 and TRIF, in order for EPS to protect from inflammatory diseases (Jones et al., 2014; Kalinina et al., 2021; Paik et al., 2020). TLR4 is a well-characterized receptor for bacterial molecules, particularly lipopolysaccharide (LPS) (Lu et al., 2008; Visintin et al., 2006). TLR4 signaling is complex and generally requires initial binding of its ligand to a coreceptor (Visintin et al., 2006). For example, LPS first binds to the soluble LPS-binding protein, which will deliver it to CD14. CD14 then transfers LPS to the TLR4-MD2 complex for initiation of intracellular signaling through TIR. The adaptor protein MyD88 amplifies the signal and leads to downstream

activation of the transcription factor NF-κB, which drives the expression of pro-inflammatory cytokines (Lu et al., 2008). Although EPS has been shown to require TLR4 signaling, it is unclear how activation of a pathway well-used by the proinflammatory molecule LPS would limit inflammation. Moreover, TLR4 is not known to bind ligand directly (Visintin et al., 2006); hence, it is unknown which receptor or receptors directly binds to EPS to activate TLR4 signaling downstream. Overall, many mechanisms both *in vivo* cell-cell interaction and *in vitro* intracellular signaling are yet to be determined. Better understanding of how the bacterial molecule EPS signals to induce an anti-inflammatory response in the host will help make better use of the probiotic bacterium *B. subtilis* and its postbiotic molecule EPS in disease settings.

Bacillus subtilis **and Exopolysaccharide in Breast Cancer**

It is virtually unknown the role of the commensal bacterium *B. subtilis* in breast cancer. Nonetheless, there are a few related clues from the literature. Urbaniak et al reported an increased abundance of *Bacillus* in breast tumors compared to healthy breast tissues (Urbaniak et al., 2016). On the other hand, the gut commensal *B. subtilis* is the primary producer of the serine protease subtilisin, which is the equivalent of chymotrypsin in mammals. Subtilisin was shown to deplete the tumor suppressor proteins Deleted in Colorectal Cancer (DCC) and neogenin in breast cancer cells, leading to enhanced migration (Forrest et al., 2016). Thus, the presence of subtilisin in the human gut, either from *B. subtilis* colonization or environmental/dietary exposure, is thought to be a contributing factor in cancer development (Stone & Darlington, 2017). Colonization by a single procarcinogenic bacterium enterotoxigenic B*acteroides fragilis* (in the gut or mammary ducts) was reported to promote breast tumorigenesis and metastatic progression (Parida et al., 2021). This was mediated by the secretion of the *B. fragilis* toxin

which activated both the β-catenin and Notch1 axis in breast cancer cells (Parida et al., 2021). Taken together, these data suggest that presence of certain bacteria either in the breast microbiome or distal gut may promote breast cancer development.

A Pubmed search using the key words "*bacillus subtilis*" and "breast cancer" returned only a handful of relevant publications. These *in vitro* studies all reported the antitumor activities of surfactin isolated from various *B. subtilis* strains. Although *B. subtilis* lipopeptides including surfactin, iturin, and fengycin have been reported to possess anti-tumor activities *in vivo* and *in vitro*, surfactin seems to be the only one with activities against breast cancer tested thus far (Zhao et al., 2017). Surfactin, a cyclic lipopeptide produced by *B. subtilis*, was shown to suppress TPA-induced breast cancer invasion by inhibiting MMP-9 expression and reducing the activation of the NF-κB, AKT and ERK signaling pathways (Park et al., 2013). Surfactin also induced apoptosis in MCF-7 breast cancer cell through a ROS/JNK-mediated mitochondrial/caspase pathway (Cao et al., 2010). Lee et al reported similar antiproliferative effects on MCF-7 cells *in vitro* by surfactin isolated from *B. subtilis* CSY191 strain found in Korean soybean paste (Lee et al., 2012). Another *in vitro* study found surfactin from the *B. subtilis* 573 strain also inhibited the growth of breast cancer cells T47D and MDA-MB-231 by inducing G1 cell cycle arrest (Duarte et al., 2014). Crudely, a mixture of lipopeptides produced by the *B. subtilis* strain HSO121 was found to alter the cellular fatty acid composition of the cell membrane and induced apoptosis of the human breast cancer cell line Bcap-37 in a dose-dependent manner *in vitro* (Liu et al., 2010). Similarly, a crude extract from a marine *B. subtilis* NMK17 strain also induced apoptosis in MCF-7 cells *in vitro* by caspase-3 activation (Sirpu Natesh et al., 2018).

A search using terms “exopolysaccharide” and “breast cancer” yielded merely 27 hits. In general, a number of exopolysaccharides produced by various bacteria were tested for their anti-tumor activities *in vitro*. The majority of EPS studied are from probiotic lactic acid bacteria (LAB). EPS from LAB were found to promote breast cancer cell (MCF-7) apoptosis and cell cycle arrest, as well as having anti-inflammatory effects (J. Wu et al., 2021). A few studies isolated EPS from marine *Bacillus* (including *Bacillus* spp. NRC5 and *B. velezensis*) and tested their antitumor activities against the breast cancer cell line MCF-7 *in vitro* (Ibrahim et al., 2020; Mahgoub et al., 2018). EPS from *Bacillus* spp. NRC5 or *B. amyloliquefeciens* 3MS 2017 exhibited anti-breast-tumor activity in mice and rats (Ibrahim et al., 2020; Mohamed et al., 2021). However, nothing was found regarding *B. subtilis* EPS in breast cancer. These highlight that *B. subtilis* and its exopolysaccharide are severely understudied, especially in the context of breast cancer.

Concluding Remarks

Breast cancer is a heterogeneous disease. A surprising 85% of breast cancer arises *de novo* in people with no genetic or familial history of breast cancer, suggesting a role for environmental factors in breast cancer development. Recent development in the study of the human microbiome postulates that dysbiosis in the gut or the breast microenvironment could play a pathological role in breast cancer. In fact, many well-established risk factors for breast cancer involve an altered microbiome. Breast tumors also possess a distinctive microbiome, that correlates with breast cancer subtype, disease severity, prognosis and treatment response. It is thought that the microbiome regulates growth of breast cancer via modulation of estrogen metabolism, the immune system, and secretion of bioactive metabolites. Particularly,

colonization of certain bacteria (*B. fragilis*) were shown to drive breast tumorigenesis and metastasis via secretion of its toxin. Bacteriotoxin (colibactin produced by *E. coli* isolated from breast tumor) can induce DNA damage and is thought to drive breast carcinogenesis by causing genomic instability. These are one of the very few examples thus far of how bacteria and the molecules they produce may contribute to breast cancer development. Much of the different microbial drivers and their mechanisms of actions remain to be discovered. Thus, now is an exciting time to open a new horizon toward the functions of bacteria cohabiting our body. Deeper understanding of how these bacteria influence breast cancer etiology will hopefully lead to better prevention strategies and novel therapies for breast cancer.

Our group is interested in studying the commensal bacterium *Bacillus subtilis* that is found in the human gut as well as ubiquitously in the environment. *B. subtilis* is well-studied as a probiotic bacterium, and the Knight laboratory has further identified the active molecule responsible for protection against inflammatory diseases to be the exopolysaccharide (EPS). Systemic administration of EPS is able to protect against a number of T-cell mediated inflammatory diseases, via modulation of TLR4 signaling on myeloid cells to generate anti-inflammatory effects. Although consumption of *B. subtilis* as a probiotic is considered safe in immunocompetent people, and EPS has been proposed for the treatment and prevention of inflammatory diseases, it was unknown whether EPS had any direct effects on breast cancer cell growth. There was an absolute black hole of information regarding *B. subtilis* or *B. subtilis* EPS on breast cancer development. Thus, this study was the first to investigate the effect of *B. subtilis* EPS on breast cancer. We examined effects of EPS treatment on breast cancer cells *in vitro* and *in vivo* across multiple phenotypes, including proliferation, migration, and survival of cancer

stem cells. Intracellular mechanisms of EPS were also investigated. The goal was to determine the mechanism by which a probiotic bacterial molecule influences growth properties of breast cancer cells and to use this knowledge to optimize the use probiotics/postbiotics to benefit breast cancer prevention and treatment.

CHAPTER TWO

METARIALS AND EXPERIMENTAL METHODS

Cell Culture

MCF-7 WS8, T47D A18, MDA-MB-231, MDA-MB-453, MDA-MB-468, ZR-75-30, HCC1428, and BT549 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). HEK293T cells were a gift from Dr. Adrash Dharan. MCF-7, T47D, ZR-75-30, MDA-MB-231, BT549, and HCC1428 cell lines were grown in antibiotic-free Roswell Park Memorial Institute Medium (RPMI-1640, Thermo Fisher Scientific, Waltham, MA). RPMI-1640 was supplemented with 10% Fetal Bovine Serum (FBS, Gemini Bio Products, Sacramento, CA), 2mM L-glutamine (Thermo Fisher Scientific, Waltham, MA), 100µM non-essential amino acids (Invitrogen, Carlsbad, CA), and 1mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA). For *in vivo*, CRISPR cloning, and long-term EPS treatments, T47D cells were maintained in above RPMI media supplemented with penicillin (50U/mL, Hyclone, Cat#SV30010) and streptomycin (50µg/mL, Hyclone, Cat#SV30010). HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher) supplemented with 2mM L-glutamine (Thermo Fisher A) and 100µM non-essential amino acids (Invitrogen, Carlsbad, CA). All cell lines were authenticated by short tandem repeat allelic profiling (ATCC, Manassas, VA) and maintained at below 20 passages for experiments. All cells were regularly tested for mycoplasma contamination using the MycoSensor QPCR assay kit Agilent Technologies, Santa Clara, CA). Cells were maintained in a 37^{o}C incubation chamber at a95% O_2 and 5% CO_2

Preparation of Exopolysaccharide Derived from *B. subtilis* (EPS)

EPS was isolated from *B. subtilis* DK7019 strain, provided by Dr. Daniel B. Kearns of Indiana University. This strain of *B. subtilis* was genetically modified (*sin*R ::cat *tas*A ::cat Δ*psg*B Physpank-eps) to overproduce/secrete EPS under Isopropyl β-D-1- thiogalactopyranoside (IPTG)-inducible condition while lacking gamma-polyglutamic acid (γPGA). *B subtilis* bacteria were cultured in 1.5% Luria Bertani broth (LB, Miller formulation) till stationary phase (OD=0.6 – 0.7), then grown as a lawn for 4 hours on 1.5% Luria Bertani agar plates (LB, Miller formulation) with 0.1M IPTG. Bacterial supernatant was collected in a digest solution (0.45% NaCl, 50μg/mL DNase and 30μg/mL RNase) and centrifuged at 9000xg at 20°C for 20 mins, twice. Supernatant was incubated in 37°C water bath for 15mins, following by digestion with 40μg/mL proteinase K at 56°C overnight. EPS was precipitated with 3-4 volume of cold ethanol at -20°C for at least 4 hours. The precipitate was pelleted by centrifugation at 13,700xg at 4°C for 30 mins, resuspended in an appropriate volume of water, and boiled in a water bath at 95°C for 10 mins. EPS was then purified by gel filtration on Sephacryl S-500 column (GE Healthcare). Carbohydrate-positive fractions were identified using a modified phenol sulfuric acid assay (Albalasmeh et al., 2013; Masuko et al., 2005). EPS-containing fractions were pooled and centrifuged through a Vivaspin column (Millipore, Germany) to isolate molecules larger than 30,000kDa. Finally, EPS was dialyzed using a 10K MWCO Slide-A-Lyzer (Thermo Fisher Scientific, Waltham, MA) for 3 days, and filtered sterilized using a 0.22μm PES syringe filter (Millipore, Germany). All EPS preparations were quantified for total carbohydrate concentration using a modified phenol sulfuric acid assay, assessed for the lack of protein and nucleic acid content by spectrometry, and tested for the ability to inhibit T47D proliferation prior to use.

Drugs, Antibodies and Reagents

Cerdulatinib, and TPCA-1 were purchased from Selleck chemicals (Houston, TX) and suspended in 100% DMSO to a stock concentration of 1mM, which were stored at -80^{o}C. These stock solutions were diluted in growth medium to obtain a working concentration of 1µM. Recombinant human TNFα protein with carrier (Cat # 210-TA-020), neutralizing anti-TNFα antibody (Cat # MAB610-100), and neutralizing anti-TNFRI antibody (Cat # MAB225-100) were purchased from R&D Systems. Recombinant human IFNɣ protein was obtained from CellGenix (Cat# 1425-050).

Matrigel Basement Membrane Matrix was purchased from Corning (Tewksbury, MA, Cat# 354234) for *in vivo* experiments. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. It mainly contains laminin, collagen IV, heparan sulfate 76 proteoglycans, entactin/nidogen, and growth factors (TGF-β, EGF, FGF, insulin-like growth factor, tissue plasminogen activator, and other growth factors which occur naturally in the EHS tumor).

Antibodies used for flow cytometry included: PE anti-human TLR4 antibody (Biolegend, Cat# 312805), PE mouse IgG2a Kappa isotype control (Biolegend, Cat# 400211), biotin anti-mouse IgG2a antibody (Biolegend, Cat# 407103), PE Streptavidin (Biolegend, Cat# 405203). Live/Dead Fixable Aqua Stain Kit was used purchased from Invitrogen (Cat# L34957).

Western antibodies STAT1 (#9172), Phosphorylated STAT1 (Tyr701, #7649), STAT3 (#9132), Phosphorylated STAT3 (Tyr705, #9131), P38 (#9212), Phosphorylated P38 (Thr180/Tyr182, #4511), P65 (#4764), Phosphorylated P65 (Ser536, # 3033) Phosphorylated IkBα (Ser32, #2859), Phosphorylated IKKα/ß (Ser176/180, #2697), IKKα (#), and RelB (#4922)

were purchased from Cell Signaling Technologies (Danavers, MA). Loading control β-Actin (A5441) was purchased from Sigma Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated secondary antibodies, including anti-rabbit (#7074) and anti-mouse (#7076), were purchased from Cell Signaling Technologies.

Lysate Preparation and Western Blot Analysis

Following experimental treatment, 200,000 cells/well were plated in a 12-well culture treated plate overnight. Cells were treated with PBS or EPS for indicated times, then the plate was placed on ice for lysate collection. Cells had their media aspirated, washed with cold PBS twice, and finally lysed in 150µl of Triton X-100 lysis buffer containing 50mM HEPES pH 7.4, 1% Triton X-100, 150mM NaCl, 5mM EDTA, 1mM Na3VO4, 10mM NaF, 1mM PMSF, protease inhibitor cocktail (Thermo Scientific, Cat#32963). Cell lysate were scraped, collected in an Eppendorf tube, and incubated on ice for 20 mins. Next, lysates were sonicated for 10 seconds at 20% amplitude, twice using the Sonic Dismembrator (Model 100, Thermo Fisher Scientific, Waltham, MA). 2µl of each sample lysate was used to determine the protein concentration using the BCA protein assay according to manufacturer's protocol (Thermo Fisher Scientific, Cat # 23225). The BCA plate was incubated at 37 ^{0}C for 30 minutes, then each well's absorbance at 562nm (A562) was measured on a 96-well plate fluorescent plate reader. Protein concentrations were calculated based on the linear regression of the protein standards: y=mx+b or sample protein concentration (x) = [(A562(y) – b)/m]. 20-30µg aliquots of lysate were prepared using 2X or 4X Laemmli buffer (BioRad, Hercules, CA, Cat # 1610737/1610747) and β-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA, Cat# BP-176-100).

Before running on western get, samples were denatured for 10 minutes at 95^0C. 20-30µg of lysates were separated on a 10% SDS-PAGE gel buffered with 8% tris-glycine. HiMark Prestained protein standard (Thermo Fisher Scientific, Waltham, MA, Cat# LC5699) was used as molecular ladder. Proteins were run at 150V for 60 minutes, and transferred to a nitrocellulose membrane at 100V for 60 minutes. Following transfer, the membrane was blocked in 5% non-fat milk diluted in Tris Buffered Saline with Tween 20 (TBST = 5mM Tris-HCL, 5mM Tris-base, 150mM sodium chloride, 0.05% Tween-20 and 0.2% NP-40 at pH 8.0). Blocking was performed for 1 hour at room temperature under constant agitation. Then, primary antibody of interested was incubated with the membrane at 4^0C overnight under constant agitation. All primary antibodies were used at 1:1000 dilution in 5% bovine serum albumin (BSA) in TBST, except Phospho-P38 (1:2000 in 5% BSA) and ß-actin (1:3000 in 5% milk). The next day, the membrane was washed 3 times in 1x TBST solution for 10 minutes under constant agitation at room temperature. Then, HRP-conjugated secondary antibody was diluted in 5% milk in TBST to appropriate concentration (anti-rabbit 1:1000 and anti-mouse 1:3000) and added to the membrane. The membrane was incubated with secondary antibody for 1 hour at room temperature under agitation, then washed 3 times with TBST for 10 minutes each. Finally, proteins were detected using Enhanced Chemiluminesence (Thermo Fisher Scientific) or SuperSignal West Extended Duration substrate (Thermo Fisher Scientific) at 1:1 volume. Membrane was incubated with above substrate for a few minutes. Stained bands were visualized with recommended exposure time on ProteinSimple machine (Biotechne, San Jose, CA). Membranes were stripped and re-probed multiple times. To strip, the membrane was first washed in TBST for 10 minutes, then stripped twice in mild stripping buffer (1L of buffer at pH 2.2

containing 15g glycine, 1g SDS, and 10mL Tween 20) for 5 minutes at room temperature under constant agitation. Then the membrane was washed twice in PBS for 10 minutes, and then twice in TBST for 5 minutes. After blocking in 5% milk for an hour, the membrane was ready for re-probing with primary antibody.

Cell Growth Assays

Growth Curve Assay

50,000 cells were seeded in triplicate in a 6-well tissue culture plate and allowed to adhere overnight. Cells were treated with either 5µg/mL of EPS or equivalent volume of sterile PBS, and media was changed every other day. Separate wells were plated to count the number of live cells following treatment on day 2, 4 and 6. Briefly, cells in each well were trypsinized, individualized and 10µL of this cell mixture was added to 10uL of trypan blue. Live cells were counted on a hemocytometer.

Total Cell Growth Assay

20,000 cells were seeded in triplicate in a 12-well tissue culture plate and allowed to adhere overnight. Cells were treated with either 5µg/mL of EPS or equivalent volume of sterile PBS. If applicable, cells were pretreated with stated concentrations of inhibitors for 30mins prior to EPS/PBS treatment. Media was changed every other day. On day 6 following treatment, the number of live cells were counted via trypan blue exclusion. Briefly, 10µL of individualized cell mixture was added to 10uL of trypan blue and live cells were counted on a hemocytometer. Fold over seeding cells were calculated as (number of live cells on day 6)/(number of live cells plated on day 0 or 20,000 cells).

XTT Proliferation Assay

2500 live cells were plated into a flat-bottom 96-well tissue culture plate to adhere overnight. Cells were treated with either PBS or increasing concentrations of EPS (0 – 10,000 ng/mL), with n=6 wells per treatment. Media was changed every other day. On day 6, media was aspirated and 150uL of working XTT solution containing 0.5 mg/mL XTT (Goldbio, Cat# X-200-100) and 3.75µg/mL Phenazine methosulfate (Sigma, Cat # P9625-1G) in phenol-red free RPMI. Plate was covered in aluminum foil and incubated at 37ºC for 2h. Absorbances at 450nm (A450) and 690nm (A690) were measured using a plate reader. To calculate corrected absorbance, we subtracted (A450- A690) of each sample with that of a blank well containing XTT solution only. Percent proliferation was calculated as [(Corrected absorbance of EPS sample/ Corrected absorbance of PBS sample)*100]. Data were graphed as log(EPS concentration) versus Percent Proliferation. The log(inhibitor) vs response – Variable slope (four parameters) model on GraphPad Prism (San Diego, CA) was used to determine the IC50 (inhibitory concentration at 50%).

Cell Cycle Analysis

100,000 cells were plated in triplicate in a 12-well tissue culture plate to adhere overnight. Cells were pretreated with stated concentrations of inhibitors or DMSO for 30mins if applicable, following by treatment with either 5µg/mL of EPS or equivalent volume of sterile PBS for 24h. Cells, media, PBS wash, and trypsin solution were collected into a flow-activated cell sorting (FACS) tube and centrifuged at 500xg for 5mins. Cell pellet was washed in 1mL cold PBS, centrifuged, and resuspended in 400uL of ice-cold PBS. To fix cells, 800µL of ice-cold 100% ethanol was added drop-wise under slow vortexing. Cells were stored at -20ºC for at

least 2 hours. On the day of analysis, cells were allowed to equilibrate to room temperature, resuspended and centrifuged at 500xg at 4^{o}C for 5 mins. Cells were washed once in 1mL cold PBS, and resuspended in 150µL of staining solution containing 50µg/mL of propidium iodide (Sigma) and 10µg/mL of RNAseA in PBS. Tubes were covered in aluminum foiled and incubated for 1h at 37^{o}C. Cell cycle analysis was conducted using LSRFortessa or FACSCantoII flow cytometers (BD Biosciences) according to the manufacturer's instructions (Cell Signaling Technology. Data was analyzed using Cell Cycle model on FlowJo V10 (BD Biosciences).

Cell Death Analysis

100,000 cells were plated in triplicate in a 6-well tissue culture plate to adhere overnight. Cells were pretreated with either 5µg/mL of EPS or equivalent volume of sterile PBS for 3 days with no media change. When cells reached 80-90% confluency on the day of analysis, cells along with media, PBS wash, and trypsin solution were collected into a flow-activated cell sorting (FACS) tube and centrifuged at 1200 RPM at room temperature for 5mins. Cells were washed with cold PBS twice, and resuspended in 1mL of 1X binding buffer (10mM HEPES/NaOH, pH7.4, 140mM NaCl, 2.5mM CaCl2, 556454, BD biosciences, San Jose, CA). Live cells were counted using trypan blue exclusion method. 100,000 cells were transferred to a new FACS tube, centrifuged and resuspended in 100µL of 1X binding buffer (BD Biosciences) containing 5µL of FITC-Annexin V (Cat# 556420, BD Biosciences, San Jose, CA) and 5µL of 7-AAD (BD Pharmingen, Cat#51-68-98E). Cells were incubated in the dark at room temperature for 15 mins, followed by addition of 400µL of 1X binding buffer (BD Biosciences). Cells were analyzed within 1 hour on the LSRFortessa or FACSCantoII flow cytometers (BD Biosciences)

according to the manufacturer's instructions (BD Biosciences). Data was analyzed with gating strategies to exclude debris on FlowJo V10 (BD Biosciences).

TLR4 Surface Marker Staining

750,000 cells were plated in a 10cm^2 tissue culture dish to reach 80-90% confluency. Media was removed and 5mL of 1X citrix saline (135mM KCL and 15mM sodium citrate) was added. Cells were incubated at 37^{o}C for 10 mins until all cells lifted off the plates. Cells were individualized and 5mL of PBS was added. Cells were centrifuged at 1200 RPM for 5 mins and washed with PBS and counted. 1 million live cells were transferred to a new FACS tube and washed in FACS buffer (3% FBS in PBS). Cells were stained with 50µl of staining solution containing 0.2µL of Live/Dead Fixable Aqua (Invitrogen (Cat# L34957) and either 5µL of PE anti-human TLR4 antibody (Biolegend, Cat# 312805) or PE mouse IgG2a Kappa isotype control (Biolegend, Cat# 400211). Cells were stained in the dark on ice for 20 mins. Cells were washed with 1mL of FACS buffer. Cells were stained with 50µL of secondary antibody solution containing 2µL of biotin anti-mouse IgG2a antibody (Biolegend, Cat# 407103) for 30 mins on ice. After washing with FACS buffer, cells were stained with 50µL of solution containing 2µL of PE Streptavidin (Biolegend, Cat# 405203) for 15 mins on ice. After final washing in FACS buffer, cells were resuspended in 300µL of FACS buffer and analyzed on the LSRFortessa or FACSCantoII flow cytometers (BD Biosciences). Data was analyzed on FlowJo V10 (BD Biosciences).

Cytokine Bead Array

10,000 cells were plated in duplicate into a 96-well U-bottom tissue culture plate to adhere overnight. Then cells were treated with either 5µg/mL of EPS or equivalent volume of

sterile PBS for 20mins, 2.5h or 22.5h. At collection time, 50µL of supernatant was collected and centrifuged at 300xg for 5mins. Then 30µL of supernatant was transferred to a new Eppendorf tube and stored at -80^{o}C until analysis. Levels of cytokines in supernatant were measured using the human Th Cytokine Panels Version 2 (Biolegend, Cat# 741028) according to manufacturer's specifications. These cytokines included Interleukin-5 (IL-5), Interleukin-13 (IL-13), Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interleukin-9 (IL-9), Interleukin-10 (IL-10), Interferon-gamma (IFN-γ), Tumor Necrosis Factor (TNF), Interleukin-17A (IL-17A), Interleukin-17F (IL-17F), Interleukin-4 (IL-4), Interleukin-22 (IL-22). Samples were analyzed using FACSCantoII flow cytometer (BD Biosciences) and data were analyzed using Legendplex v8.0 (BioLegend).

Mammosphere Forming Assay

Preparation of Mammosphere Medium

For each batch of mammosphere media, we added 196 mL of warm DMEM-F12 medium (Gibco, Gaithersburg, MD, Cat. 11039021) to a sterile bottle containing 4 grams of methocellulose. The solution was initially stirred in a water bath at 60^{o}C for 30mins, then it was allowed to continue mixing overnight at 4^{o}C. The next day, we added 4mL B-27 supplement and 4µL recombinant hEGF (Sigma-Aldrich, Milwaukee, WI, Cat. E-9644) to the medium and stirred the solution for 30 mins at room temperature. The solution was transferred to 50mL centrifuge tubes and centrifuged at 8000 RPM in Beckman rotor for 30 mins at 4^{o}C. The supernatant was poured into 50mL conical tubes and stored at -20^{o}C until used. The mammosphere medium was thawed in a bead bath at 37^{o}C for 2-3h prior to use.

Mammosphere Forming Assay

100,000 T47D cells were plated in a 6-well tissue culture plate to adhere overnight. Then cells pretreated with stated concentrations of inhibitors or DMSO for 30 mins if applicable, following by treatment with either 5µg/mL of EPS or equivalent volume of sterile PBS for 4 days. Cells were harvested using trypsin and individualized. Live cells were counted using trypan blue and 25,000 cells were plated in 6-well ultra-low attachment plate with 3mL of mammosphere medium containing gentamycin. The whole plate was gently rocked several times to evenly distribute the cells across the well. The plate was left undisturbed in the incubator at 37^{o}C and 5% CO_2 for 7 days to allow mammospheres to form. On day 7, mammospheres were first imaged at 4X objective on a microscope. To extract mammospheres for counting, 6mL of PBS was added to the well and pipetted a few times to mix with the viscous mammosphere medium. Mixture containing mammospheres were transferred to a 15mL conical tube. This process was repeated with another 6mL of PBS. Next, this tube was weighted on a scale to estimate total volume. After inverting the tube multiple times to mix, 375µL was transferred to a 96-well plate and allowed to settle for 5 mins. Mammospheres in the 96-well plate was imaged at 4X objective along with measurement scale under a microscope. 5 pictures were taken for each well to ensure the whole well was captured. These pictures were placed into PowerPoint, and mammospheres larger than 100microns (or 50microns in some cases) were counted manually based on the scale stamped in the picture. Based on the total volume weighted, the diluted factor was calculated to estimate the total number of mammospheres present in the sample based on the mammospheres counted. Finally, percent mammosphere forming efficiency (%MFE) was calculated as [(total number of mammospheres/ 25,000 cells plated)*100].

Wound-Healing Migration Scratch Assay

200,000 cells were plated in triplicate in a 12-well tissue culture plate to adhere overnight. Cells were pretreated with stated concentrations of inhibitors or DMSO for 30mins if applicable, following by treatment with either 5µg/mL of EPS or equivalent volume of sterile PBS for 2 days until confluent. Then cells were starved in media containing 3% FBS and drug treatments overnight. Media was aspirated and 3mL of PBS added to the well. Then a 10µL pipette tip was used to scratch the confluent monolayer of cells, creating a cross shape in the well. The scratches were immediately imaged at 2 locations of the cross at 10X objective under the microscope (0 h). Media was changed to contain 3% FBS and appropriate drug treatment. At 24h and 48h following scratching, media was changed and scratches were imaged at the same location relative to the cross shape. Migration rate was quantified as open gap area using ImageJ according to Venter and Niesler protocol (Venter & Niesler, 2019). Percent wound closure was calculated as [100 - (Gap area at 24h or 48h/Gap area at 0h)*100].

Metabolic Seahorse Assay

6000 cells were plated into 96-well Agilent Seahorse XF Cell Culture Microplate (Agilent Technologies, Cat# 101085-004) to adhere overnight. Cells were pretreated with 5µg/mL EPS or PBS for 40hours, n=10 per treatment. Prior to seahorse assay, cells were changed to seahorse media without serum and incubated in a normal cell-culture incubator (37^{o}C and 5% CO_2) for 2h and then moved to an incubator without CO_2 for 1h. For acute EPS treatment, EPS was injected into the PBS-pretreated wells right before metabolic rates were measured. Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, Cat# 103015-100) were used to measure metabolic rates on a Seahorse XFe96 Analyzer (Agilent Technologies, Santa

Clara, CA) according to manufacturer's protocol. Mitochondrial respiration modulators were used at specified concentrations: Oligomycin at 1.5µM, FCCP at 0.5µM, and Rotenone/antimycin A at 0.5µM. Metabolic rates (PER glycolytic metabolism and OCR oxidative metabolism) were normalized by protein concentration measured in the well.

RNA Interference and Transfection

A pool of four siRNAs were purchased from Dharmacon GE Life Sciences (Lafayette, CO) for each of the following genes: STAT1 (ON-TARGETplus SMART pool Cat# L-003543-00-0005), JAK1 (ON-TARGETplus SMART pool Cat#L-003145-00-0005), IKK-beta (ON-TARGETplus SMART pool Cat# L-003503-00-0005), P65 (ON-TARGETplus SMART pool Cat# L-003533-00-0005). Non-targeting scrambled control siRNA (SCBi) was purchased from Qiagen (Germantown, MD). siRNAs were reconstituted in siRNA Diluent Buffer (10mM Tris-HCl, pH 8.0, 20mM NaCl, 1mM EDTA) at 10µM working solution and stored at -20°C. The transfection reagent Lipofectamine RNAiMAX (Cat# 13778150) was purchased from Thermo Fisher Scientific (Waltham, MA) and used at a ratio of 1:1 with 50nM of appropriate siRNA according to the manufacturer's protocol. 1.2 million T47D cells were plated in a 10-cm^2 tissue culture overnight. The iMAX solution was prepared by adding 40µL of RNAiMAX to 460µL of Opti-MEM (per transfection) in a 1.5mL Eppendorf tube. In parallel, 40µL of siRNA was added to 460µL of Opti-MEM per transfection in separate tubes. Solutions were incubated for 5 minutes. After incubation, 500µL of iMAX solution was then added to each siRNA condition and allowed to incubate for 20 minutes at room temperature. The adherent cells were then washed with PBS 2X and 7mL of RPMI was added to each plate followed by 1000µL of the

siRNA + iMAX solution in a drop-wise fashion. Plates were gently swirled to mix the solution and incubated at 37°C for 48 hours before splitting into experiments.

Reverse Transcription and Real-Time Polymerase Chain Reaction

2 X 10^5 T47D cells were plated in 6 cm^2 dishes and allowed to adhere overnight. The following day, cells were treated with their respective conditions and incubated at 37°C for 24h. Plate was placed on ice, and cells were washed 2X with cold 1X PBS. Then 300µL of TRIzol (Thermo Fisher Scientific, Waltham, MA) was added to each well to resuspend the cells. The cell mixture was collected in a 1.5mL Eppendorf tube and 50µL of 1- bromo-3-chloropropane (BCP) was added to each sample (Sigma Aldrich, St. Louis, MO) and vortex for 10 seconds. Samples were centrifuged at 15,000 RPM for 15 minutes at 4°C. The clear, aqueous phase was carefully isolated and transferred to a new 1.5mL Eppendorf tube. To this tube, equal volume of 100% EtOH was added and the solution was gently vortexed. The total RNA from the sample was collected using the RiboPure Kit (Thermo Fisher Scientific). The sample was passed through a filter cartridge by centrifugation at 16,000xg for 30 seconds at room temperature. Each sample was washed with 400µL of Direct-zol RNA Prewash and flow-through was discarded. 5µl of DNAse I in 75µl of DNAse digestion buffer (RiboPure™ Kit, Thermo Fisher Scientific) was added directly onto the column. After 15min of incubation at room temperature, 400µL of Direct-zol RNA Prewash was added. The column was centrifuged and flow-through discarded. Samples were washed again with 200µL of Direct-zol RNA Prewash and spun for 30 seconds. Finally, 500µL of RNA wash buffer was added and column was spun for 2min. The tubes were spun one more time at 16,000xg for 1 minute to discard any excess buffer. The column containing RNA was moved to a new 1.5mL Eppendorf tube, and 30µL of DNase/RNase-free

water was gently added to the column membrane. Samples were incubated at room temperature for 1 minutes and spun a final time at 16,000xg for 1 minute to elute RNA. Total RNA quality and quantity was determined by measuring the UV absorbance at 260nm using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

Isolated RNA was converted to cDNA using the TaqMan Reverse Transcriptase Kit (Applied Biosystems, Ford City, CA) according to manufacturer's protocol. Briefly, 0.5µg of RNA was added to a 50µL reaction volume consisting of 1X RT buffer, 5.5mM MgCl2, 500µM dNTPs, 2.5µM random hexamers, 0.4U/µL RNase inhibitor, and 1.25U/µL RT enzyme (MultiscribeTM Reverse Transcriptase Enzyme, Applied Biosystems). The reverse transcriptase reaction was run in a thermocycler as followed: 10 minutes at 25°C, 30 minutes at 48°C, 5 minutes at 95°C, 60 minutes at 25°C, and held at 4°C until use.

RT-PCR was performed using the iTaqTM SYBR® Green Enzyme Supermix with ROX (BioRad, Hercules, CA) according to manufacturer's protocol. In a 96-well optical PCR plate, 1.25µL of cDNA was added to 11.25µL of master-mix solution containing 50µM forward and reverse primers, RNase-free water, and 2x SYBER Green. Each condition was performed in duplicate. The RT-PCR reaction was ran using a StepOnePlus thermocycler (Applied Biosystems, Foster City, CA) as followed: initial denature at 95°C for 10 minutes, PCR cycling for 10 seconds at 95°C for 40 cycles, and annealing for 45 seconds at 60°C. Melt curves were performed to ensure proper amplicon formation and the average cycle threshold (CT) was used to determine the relative gene expression for each experimental condition. The CT value was calculated as the number of cycles necessary for the fluorescent signal to overcome the background level, or threshold, of fluorescent signal. CT values were normalized to the

housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT), an endogenous control, to discern ΔCT. ΔCT for gene of interest= CT (experimental gene)-CT (HPRT). ΔΔCT was calculated by normalizing the ΔCT values to a control sample. Relative quantification (RQ) was calculated using the $2^{-\Delta\Delta CT}$ method to determine relative fold increases or decreases in transcript compared to the designated control sample. The PCR primers used for the detection of specific transcripts are shown in **Table 2** below.

Table 2. RT-PCR Primer List and Sequences.

Primer Name	**Sequence**
HPRT Forward	ATGAACCAGGTTATGACCTTGAT
HPRT Reverse	CCTGTTGACTGGTCATTACAATA
STAT1 Forward	AGGAAGACCCAATCCAGATGT
STAT1 Reverse	CAGGCTCTTGATTTCATGCTC
IRF1 Forward	GATGACCACAGCAGCTACACA
IRF1 Reverse	TTCCCTTCCTCATCCTCATCT
CDKN1A (P21) Forward	ACTTTGTCACCGAGACACCAC
CDKN1A (P21) Reverse	AGGTCCACATGGTCTTCCTCT
TNF Forward	ATGAGCACTGAAAGCATGATCC
TNF Forward	GAGGGCTGATTAGAGAGAGGTC

RNA Sequencing and Pathway Analysis

4 X 10^5 T47D A19 cells, 1 X 10^6 MCF7 WS8 cells, 2 X 10^5 MDA-MB-231 cells, or 8 X 10^5 MDA-MB-468 cells were plated in 10 cm^2 dishes to adhere overnight. The following day,

cells at <70% confluence were treated with either 5µg/mL EPS or equal volume of PBS and incubated at 37°C for 24 hours. Each condition was performed in 3 biological replicates. Total RNA was extracted using the RNeasy mini Kit (Qiagen, Germantown, MD) and sent to Novogene for RNA-library preparation and RNA-sequencing. Data analysis was also performed by Novogene. In addition, 290 genes were identified as being upregulated by EPS only in sensitive (T47D and MDA-MB-468) and not resistant cell lines (MCF7 and MDA-MB-231). Pathway analysis was conducted on this set of genes using the Metascape pathway analysis software (https://metascape.org), with pathway enrichment being plotted by p-value for the number of genes in a given Gene Ontology (GO) pathway.

CRISPR/CAS9 Genome Editing

TLR4 and STAT1 knockout T47D cells were generated using LentiCRISPRv2 (Addgene plasmid 52961), gifted from Feng Zhang (Sanjana et al., 2014). 1-2 single guide RNA (sgRNA) sequences targeting either TLR4 or STAT1 were designed using E-CRISPR (Heigwer et al., 2014). For cloning purposes, the forward sgRNA oligo was made as 5'-caccg[sgRNA sequence]-3' while the reverse oligo was made as 5'-aaac[reverse complement of sgRNA sequence]c-3'. Each pair of sgRNA oligonucleotides was cloned into the LentiCRISPRv2 backbone containing Cas9. Briefly, each sgRNA oligo was resuspended in TE buffer, and 100µM of Forward and Reserve sgRNA oligo was annealed in a 50µl reaction with water as programmed: 37°C for 30 min, 95°C for 5 min, 70°C for 30 min, 65°C for 30 min, 60°C for 30 min, 55°C for 30 min, 50°C for 30 min, 45°C for 30 min, 40°C for 30 min, 35°C for 30 min, 30°C for 30 min, 25°C for 30 min, and held at 22°C. Digestion and ligation reactions were set up as followed: 75ng LentiCRISPRv2 backbone, 1µl annealed oligos, 1µl 10X ligation buffer, 0.5µl T4 ligase, 0.5µl

BsmBI, and 6µl water. The thermocycler was programmed as follows: [37°C for 10 min, 16°C for 5 min] x 10 cycles, 55°C for 20 min, 80°C for 20 min and then held at 4°C. Next, 0.4µl ligation reaction mixture was transformed into DH10B electrocompetent bacteria and plated onto LB-Ampicilin plates overnight at 37°C. A few colonies were selected, cultured, and miniprepped to collect DNA. Successful clones were tested via digestion with restriction enzymes, KpnI and AgeI, to generate a ~621bp cut from the backbone vector. Briefly, sgRNA clones were cut in 25µl reaction volume containing 0.5µg of DNA, 1µl of KpnI (NEB), 1µl of AgeI (NEB) and 1X NEB Buffer 1.1 (NEB) for 1h at 37°C. 10µl of the digested products were ran on 8% agarose gel to assess for the presence of the 621bp fragment. Selected clones were further sequenced using hU6-Forward primer (5'-GAGGGCCTATTTCCCATGATT-3') and respective reverse sgRNA oligo. Briefly, 1µg DNA and 25pmoles of each primer pair in a 15µL volume were sent to GenScript for sequencing.

For lentivirus production, HEK293T cells were plated into a 10cm^2 plate until 70% confluent. Lentiviral transfection reaction master mix containing 3.3µg of VSV-G (pCMV-VSV-G was a gift from Bob Weinberg, Addgene plasmid # 8454 (Stewart et al., 2003)), 3.3µg of psPAX2 (catalog no. 11348; Didier Trono, NIH AIDS Reagent Program) (Zufferey et al., 1998)), 3.3µg of LentiCRISPRv2 (containing the guide RNA of interest), 1mL of Opti-MEM and 22.5µl PEI was incubated at room temperature for 30mins. Then the mixture was added dropwise onto HEK293T cells containing 9mL of fresh DMEM media. Cells were incubated at 37°C for 24h before the media was changed to collection media. Viral supernatant was harvested at 48 hours post-transfection and syringe-filtered through 0.45µm filters (Millipore). For lentiviral transduction, 1.8 million T47D cells was plated into a 10cm^2 dish to adhere overnight. Then all

of the freshly filtered virus from the $10cm^2$ dish of HEK293T cells was added to the $10cm^2$ dish of T47D cells. Cells were incubated at 37°C overnight and media was changed the next day. 48 hours after transduction, 2μg/mL of puromycin (lowest doses based off of the antibiotic kill curve) was added to the media. Puromycin selection was maintained for 5-6 days, until all untransduced cells died. Following puromycin selection, single clones of transduced cells were obtained using the array serial dilution method (Wang, 2018). Briefly, 4000 of pooled transduced cells (20,000 cells/mL) were added to the first well of a 96-well plate. Then, a 1:2 dilution was serially made down the 8 wells of first column of the plate, followed by 1:2 serial dilution across the 12 wells of each row of the plate until some wells should contain single clone. Each well was observed for cell growth, and cells from wells calculated to contain <8 cells were expanded and tested for gene knockout phenotype.

STAT1 knockout phenotype was assessed via western blot for protein expression. TLR4 knockout was assessed via genomic alteration. Primers were designed to flank the genomic region targeted by TLR4_sgRNA1, which is exon1 of TLR4, to generate a 107bp amplicon (Table 3). Single clones isolated from a pooled of cells transduced with TLR4_sgRNA1 were tested for size alterations at the exon 1 site. Genomic DNA was isolated from each clone of cells using the Promega Wizard Genomic DNA Purification Kit (Promega, Cat# A1120). PCR amplification of TLR4 exons were performed in a 50μL reaction containing 100ng DNA, 0.2μM Forward and Reverse primers, 1mM MgCl2, 0.2mM dNTP, 0.5μL GoTaq Hot Start Polymerase (Promega, Cat#M500A), 1X Green GoTaq Flexi Buffer (Promega, Cat#M891A). Thermocycler was programmed as followed: 95°C for 10 min, 40 cycles of [94°C for 30 sec, 57°C for 45 sec, 72°C for 1 min], 72°C for 5 min, and held at 4°C. 20μL of PCR products were ran on a 6%

acrylamide gel at 105V for 30min. Clones of interested are those that produced amplicons having a different size than the expected amplicons of wild-type cells. A clone of TLR4_sgRNA1 transduced cells (clone F6) displayed 1 TLR4 exon1 amplicon slightly larger (108bp) and 1 amplicon smaller (81bp) than the single amplicon (107bp) seen in cells transduced with Cas9 only. Both amplicons from clone F6 and the single amplicon from Cas9-only T47D cells were sequenced to reveal the exact genomic alterations. Briefly, genomic DNA was PCR amplified using TLR4 exon1 primers as above, and all PCR products were run on a 10% acrylamide gel to separate by size. Each of the bands (~107bp for Cas9-only, 108bp and 81bp for clone F6) was cut out of the gel under Ultraviolet light. The gel fragment was crushed in 300µL of 0.3% TE buffer and allowed to dissolve overnight at 4°C. The mixture was roughly filtered to eliminate undissolved acrylamide gel, then DNA was ethanol precipitated and rehydrated in 4µL of sterile water. Each DNA fragment was cloned into a pGEM-T vector (Promega, Cat#A1360) for sequencing. Briefly, ligation reaction was set up in a 0.5mL tube at 4°C overnight containing: 20ng DNA, 1µL pGEM-T Easy Vector, 1X Rapid ligation buffer, 0.6µL T4 DNA ligase, and water to 10µL volume. The following day, 0.4µL of ligation reaction was used to transform electrocompetent DH10B cells. Cells were incubated on Ampicilin/IPTG/X-gal agar plate at 37°C overnight for blue/white selection. A few white colonies were selected, cultured, and miniprepped to collect DNA. Successful clones were tested via digestion with restriction enzyme EcoRI. Briefly, recombined plasmids were cut in a 30µl reaction volume containing 1µg of DNA, 1µl of FastDigest EcoRI (Thermo Scientific, Cat #FD0274), and 1X FastDigest Buffer (Thermo Scientific, Cat #FD0524) for 45min at 37°C. 20µl of the digested products were ran on 6% acrylamide gel to assess for the presence of the original amplicon. Clones containing the

amplicon of interested were sent for sequencing with TLR4_exon1 primers. Obtained amplicon sequences were aligned with Ensembl's TLR4 gene sequence to identify genomic alteration.

Table 3. CRISPR Primer List and Sequences.

Primer Name	**Sequence**
TLR4_sgRNA1 Forward	GATGATGTCTGCCTCGCGCC
TLR4_sgRNA1 Reverse	GGCGCGAGGCAGACATCAT
STAT1_sgRNA1 Forward	GACGAGGTGTCTCGGATAGT
STAT1_sgRNA1 Reverse	ACTATCCGAGACACCTCGTC
STAT1_sgRNA2 Forward	AAAGCTGGTGAACCTGCTCC
STAT1_sgRNA2 Reverse	GGAGCAGGTTCACCAGCTTT
TLR4_exon1 Forward	GCCATCGCTGCTCACAGAAG
TLR4_exon1 Reverse	GGTCTCACGCAGGAGAGGAA

Xenograft Tumor Growth

All animal study protocols were approved by Loyola University's Institutional Animal Care and Use Committee. Per *in vivo* experiment, about 100 million T47D cells per treatment were required. T47D cells were first expanded in 150cm^2 tissue culture treated flasks and treated with 5μg/mL EPS or equal volume of PBS for 5 days. Then 40 million EPS or PBS-treated T47D cells were transferred to a Nunc Cell Factory System (Thermo Scientific, Cat# 140004TS) with continued *in vitro* treatment for another 3 days. On collection day, cells were trypsinized, collected and washed with 1X PBS before being resuspended in Matrigel Matrix Basement Membrane Phenol-Red Free (Cat# 356237, Corning, Bedford MA) to a concentration of 4

million live cells per 100µL of Matrigel. For EPS-treated cells, EPS was also added to the Matrigel:Cell suspension to an estimated concentration of 300µg/mL. Then 100µL of Matrigel:Cell suspension was injected bilaterally into the fourth mammary fat pads of 9-10 weeks old, female, ovariectomized *Foxn1* nu/nu athymic nude mice (Envigo, IN). Mice were also implanted with a 0.3cm silastic capsule containing 17β-estradiol for a constant release of 83-100pg/mL as previously described (O'Regan et al., 1998). This estrogen capsule was replaced at 8th week when it ran out. Each mouse was tagged with an ear tag for identification and tumor tracking purposes. 4 mice each were implanted with EPS or PBS-pretreated cells followed by intraperitoneal injection with respective 50µg EPS or 100µl PBS 3 times/week. Tumor area (length x width) was measured weekly using Vernier calipers. Mice were sac on day 94 and tumors were imaged, weighted, and frozen at -80^{0}C. Tumor growth as tumor weight and tumor volume were calculated and graphed.

For the experiment with NOD.SCID mice, 100 million T47D cells per condition were grown and pretreated *in vitro* with PBS or EPS for 8 days as above. On collection day, EPS-treated cells were resuspended in Matrigel with EPS added to a concentration of 80µg/mL. 4 million cells were injected bilaterally into the fourth mammary fat pads of 9-10 weeks old, female, ovariectomized NOD.SCID mice (Envigo, IN). 5 mice were used for PBS group and 7 mice for EPS group. Mice were also implanted with an estrogen capsule and ear tag as described above. The estrogen capsule was not replaced at 8th week, but was left in place until the experiment ended. Each mouse was injected (ip) with 25µg EPS or 100µl PBS 3 times/week and tumor area (length x width) was measured weekly using Vernier calipers. Mice were sac on day 87 to assess tumor burden.

Statistical Analysis

Experiments were conducted in triplicate and repeated three independent times, with results reported as Mean ± SEM. Statistical analysis was performed and graph figures were generated using Prism Version 9 (GraphPad Software). A two-sided student's t-test was used to compare 2 groups, and P-values <0.05 were considered statistically significant. A one-way ANOVA with a post- hoc Tukey's test was used to compare multiple groups. For *in vivo* studies, tumor volumes were calculated as $[(\text{Length X } (\text{Width})^2)/2]$. Linear regression analysis was performed and the slope of tumor growth over time was used to compare the growth rate between treatment groups.

CHAPTER THREE

EXPERIMENTAL RESULTS

Characterizing the Effects of *B. subtilis* EPS on Breast Cancer Proliferation *in vitro*

The Effect of EPS on Proliferation of Breast Cancer Cells

Various exopolysaccharides produced by bacteria displays anti-cancer activities *in vitro* (Ibrahim et al., 2020; Mahgoub et al., 2018; Mohamed et al., 2021; J. Wu et al., 2021). EPS produced by *B. subtilis* has been shown to act on myeloid cells to inhibit T-cell proliferation (Jones & Knight, 2012; Kalinina et al., 2021; Paik et al., 2019; Swartzendruber et al., 2019). Thus, we initially hypothesized that EPS would inhibit the proliferation of some breast cancer cell lines in a dose-dependent manner. To assess the effect of EPS on breast cancer cell proliferation, we treated a panel of breast cancer cell lines of distinct subtypes (ER+ cell lines T47D and MCF-7, triple-negative cell line MDA-MB-468) with increasing concentrations of EPS. The XTT cell viability assay measured proliferation and/or cytotoxicity compared to PBS treated cells at day 6. We found that EPS inhibited the proliferation of both T47D and MDA-MB-468 cell lines in a concentration-dependent manner, while the MCF-7 cell line was unaffected (Figure 2). Moreover, the dose-response curve for T47D cells displayed a distinctive sigmoidal shape that is potentially suggestive of chemical inhibitors that target a single receptor (Salahudeen & Nishtala, 2017; Weiss, 1997). Proliferation of a triple-negative breast cancer cell line, MDA-MB-468, was also inhibited by EPS but at lower efficacy (maximal inhibition at 50% compared to control vs 20% in T47D cells), and the shape of the response curve suggested a

possible role for multiple pathways **(**Figure 2**)**. The inhibitory concentration at 50% (IC50) was determined to be 10ng/mL for MDA-MB-468 and 30ng/mL for T47D, with maximum inhibition achieved at approximately 1µg/mL EPS for both cell lines **(**Figure 2**)**. Hence, a concentration of 5µg/mL EPS was selected for all additional *in vitro* assays to ensure maximal effects despite variations across different preparations of EPS.

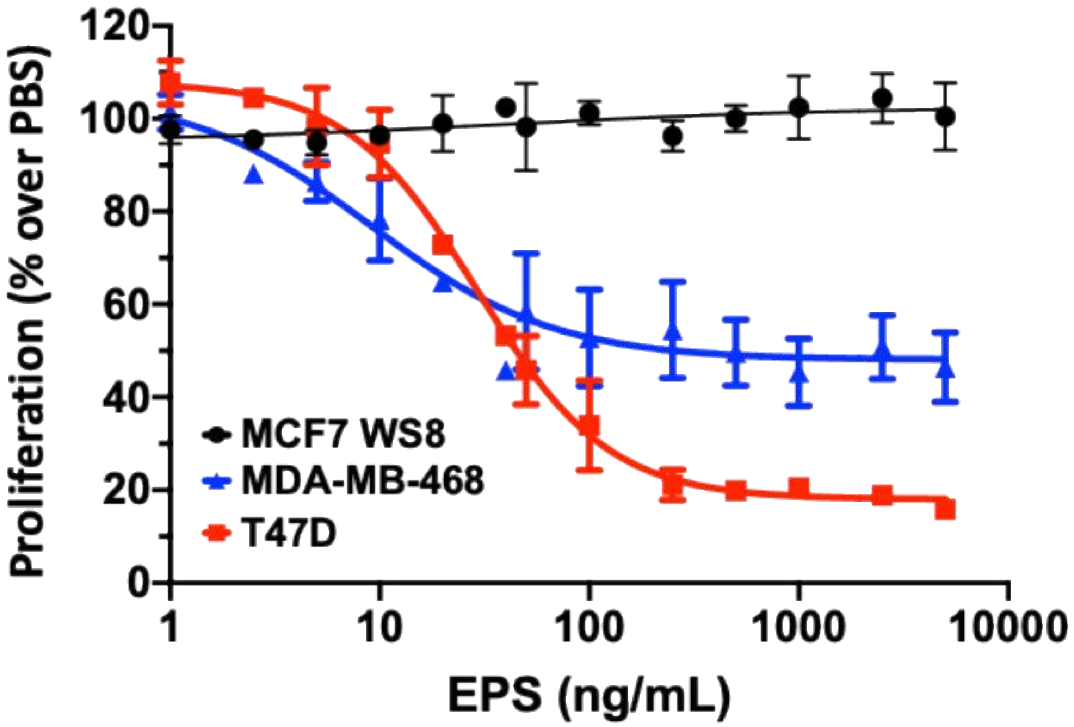


Figure 2. The Proliferation of Three Breast Cancer Cell Lines in Response to Increasing Concentrations of EPS. Three breast cancer cell lines were grown in media containing PBS or increasing concentrations of EPS for 6 days. The XTT viability assay was used to measure the number of live cells in each treatment (n=6). The PBS-treated group was set at 100% proliferation. Percent proliferation of the EPS treated groups was calculated by dividing EPS over PBS multiplied by 100. The log(inhibitor) vs response – Variable slope (four parameters) model was fitted as shown to determine the IC50 (inhibitory concentration at 50%). Data are represented as mean ± SEM of 3 independent experiments.

To further test our hypothesis that EPS inhibits breast cancer growth, we examined the effect of EPS on other breast cancer cells using a proliferation assay measuring the number of

live cells using trypan blue. Cells were grown in PBS or 5µg/mL EPS-containing medium and the number of proliferating/live cells were counted every other day for 6 days. We expected that EPS would inhibit proliferation of additional breast cancer cell lines. Of the 8 cell lines tested, one-half of them were inhibited by EPS (T47D, MDA-MB-468, HCC1428, and MDA-MB-453) while the rest were unresponsive (MCF-7, ZR-75-30, MDA-MB-231, and BT549) (Figure 3). Thus, the response to EPS is not restricted to certain breast cancer subtypes.

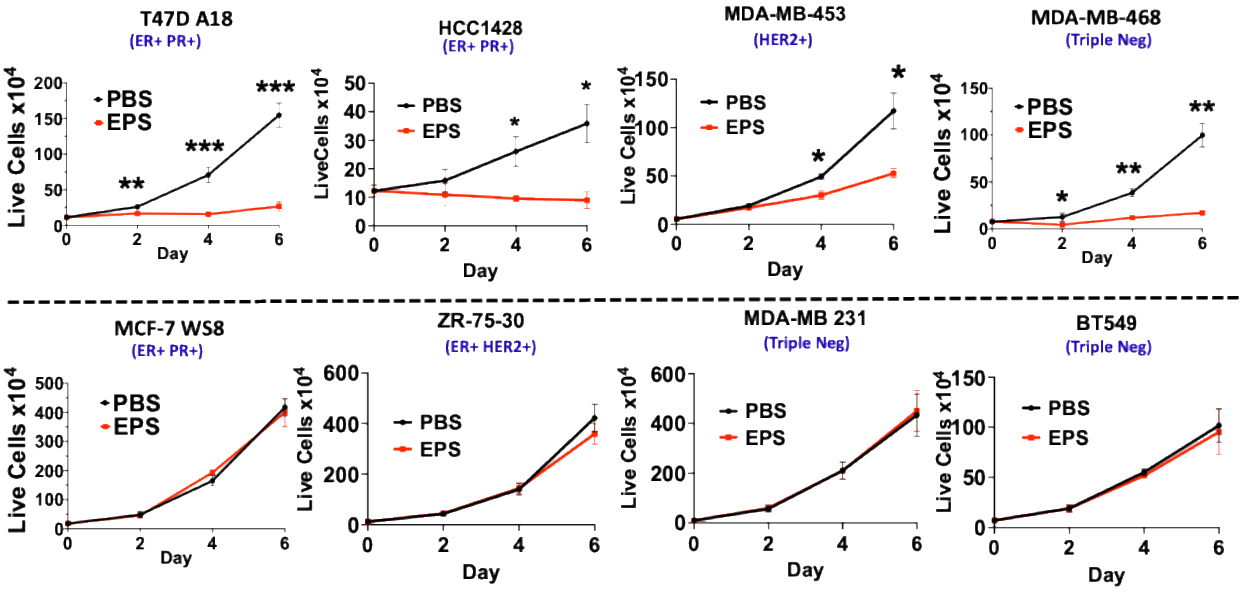


Figure 3. The Proliferation of Different Breast Cancer Cell Subtypes in Response to EPS. The proliferation rates for 8 breast cancer cell lines were measured by treating cells with PBS or 5µg/mL EPS for 6 days. Live cells were counted by trypan blue exclusion on a hemocytometer. Data are represented as mean ± SEM of 3 independent experiments performed in triplicate. A Student's *T*-test was calculated to determine statistical significance between EPS and PBS. * $P \leq 0.05$, ** $P \leq 0.01$ *** ≤ 0.001.

Cell Cycle Progression and Cell Death

Inhibition of cell proliferation is either through increased cell death or cell cycle arrest. Knowing which pathway EPS targets would help to narrow the mechanism utilized by EPS. Since EPS is able to inhibit cell proliferation, we hypothesized that EPS induces cell death and/or

cell cycle arrest in the responsive breast cancer cell lines. To test these two possibilities, we measured cell death using Annexin V and 7-AAD staining and analyzed cell cycle progression using propidium iodine staining followed by flow cytometry. Each of the four responsive cell lines (T47D, MDA-MB-468, HCC1428, and MDA-MB-453) was treated with PBS or 5µg/mL EPS for 24 hours to assess cell cycle progression or 72 hours for cell death. We expected to see either cell death and/or cell cycle arrest in the G1/G0 or G2/M phase in each of the responsive cell line. EPS increased the percentage of T47D cells in the G1/G0 phase and decreased cells in the S phase (Figure 4A). EPS had little effect on cell death of T47D cells (Figure 4B). The other 3 cell lines (MDA-MB-468, HCC1428, MDA-MB-453,) displayed minimal change in cell cycle progression in response to EPS (Figure 5A, 6A, 7A). However, EPS increased cell death of these cells, with 2 to 3-fold increase in apoptotic Annexin V+ cells (Figure 5B, 6B, 7B). The cell death analysis was performed in triplicate for the MDA-MB-453 cell line and results trended in the same direction as the other cell lines (Figure 7B). Given the heterogeneity of breast cancer cell lines, it was not surprising that EPS induced cell cycle arrest in some cell lines and cell death in others.

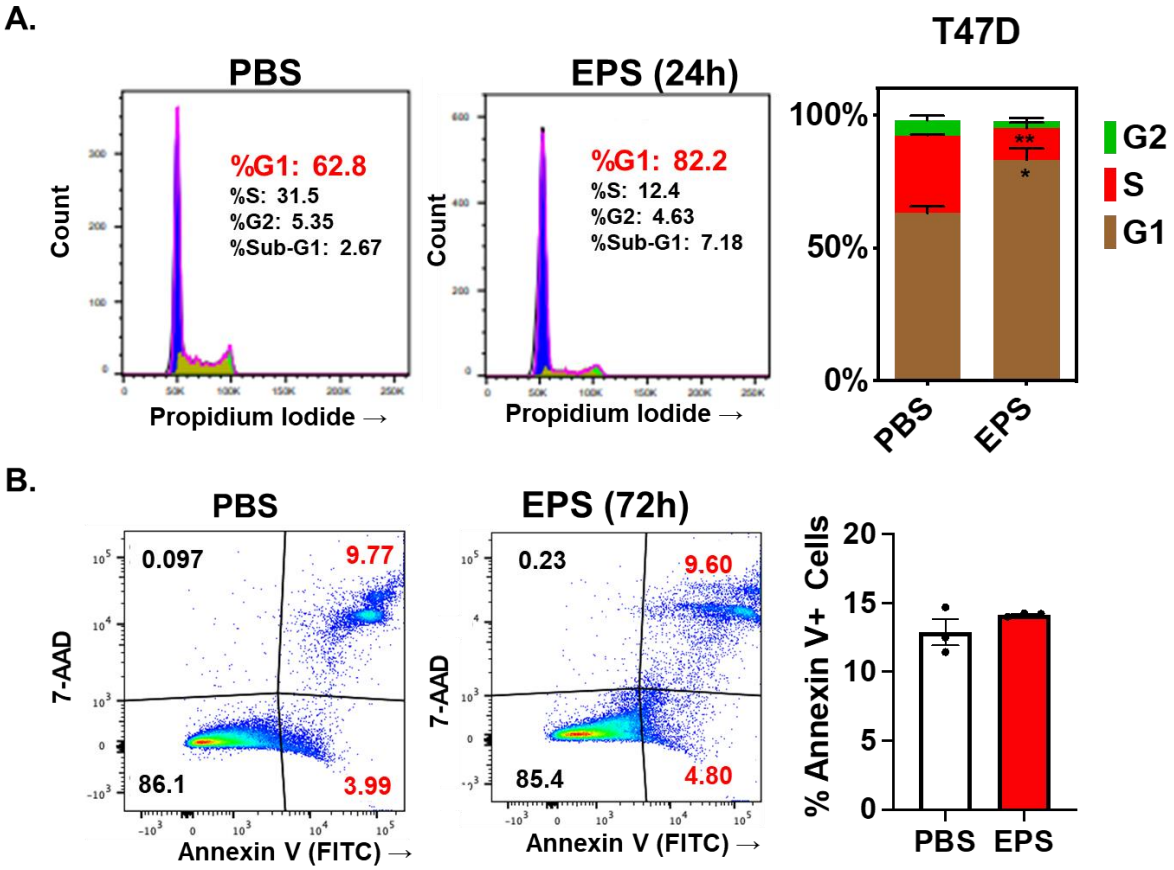


Figure 4. Effects of EPS on Cycle Cell and Cell Death in T47D Cells. A. T47D cells were treated with PBS or 5µg/mL EPS for 24h and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Representative flow plots (left) and graph (right): Data are represented as mean ± SEM of 3 independent experiments performed in triplicate, with t-test * P ≤ 0.05, ** ≤ 0.01. **B.** T47D cells were plated in triplicate in a 6-well plate and treated with PBS or 5µg/mL EPS for 3 days. Apoptosis was assessed by staining with Annexin V and 7-AAD and analyzed by flow cytometry. Representative flow plots (left) and graph (right): Percent of Annexin V positive cells (including 7-AAD positive cells) as mean ± SEM of 3 independent experiments performed in duplicate, with the t-test not being significant.

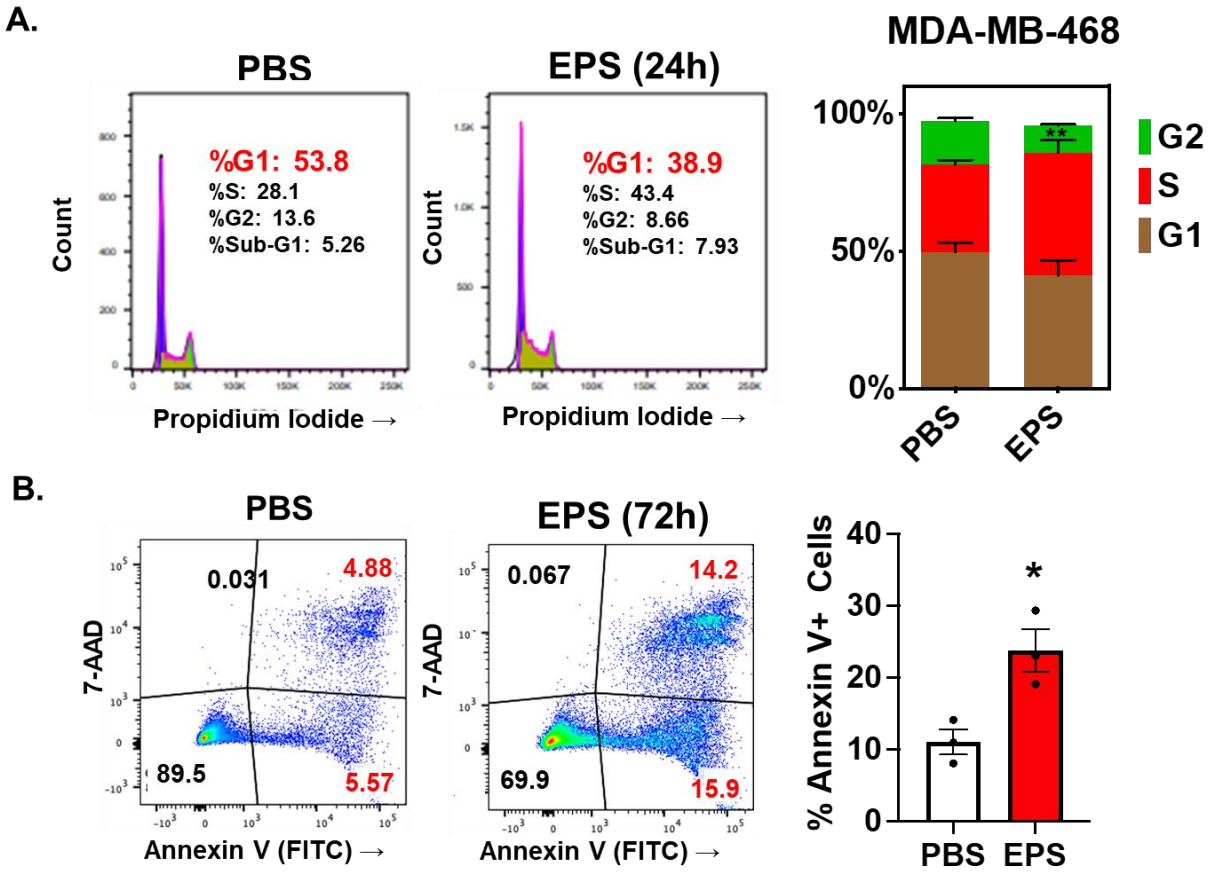


Figure 5. Effects of EPS on Cycle Cell and Cell Death in MDA-MB-468 Cells. A. MDA-MB-468 cells were treated with PBS or 5µg/mL EPS for 24h and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Representative flow plots (left) and graph (right): Data are represented as mean ± SEM of 3 independent experiments performed in triplicate, with t-test ** ≤ 0.01. **B.** MDA-MB-468 cells were plated in triplicate in 6-well plate and treated with PBS or 5µg/mL EPS for 3 days. Apoptosis was examined by staining with Annexin V and 7-AAD and analyzed by flow cytometry. Representative flow plots (left) and graph (right): Percent of Annexin V positive cells (including 7-AAD positive cells) as mean ± SEM of 3 independent experiments performed in duplicate, with t-test * P ≤ 0.05.

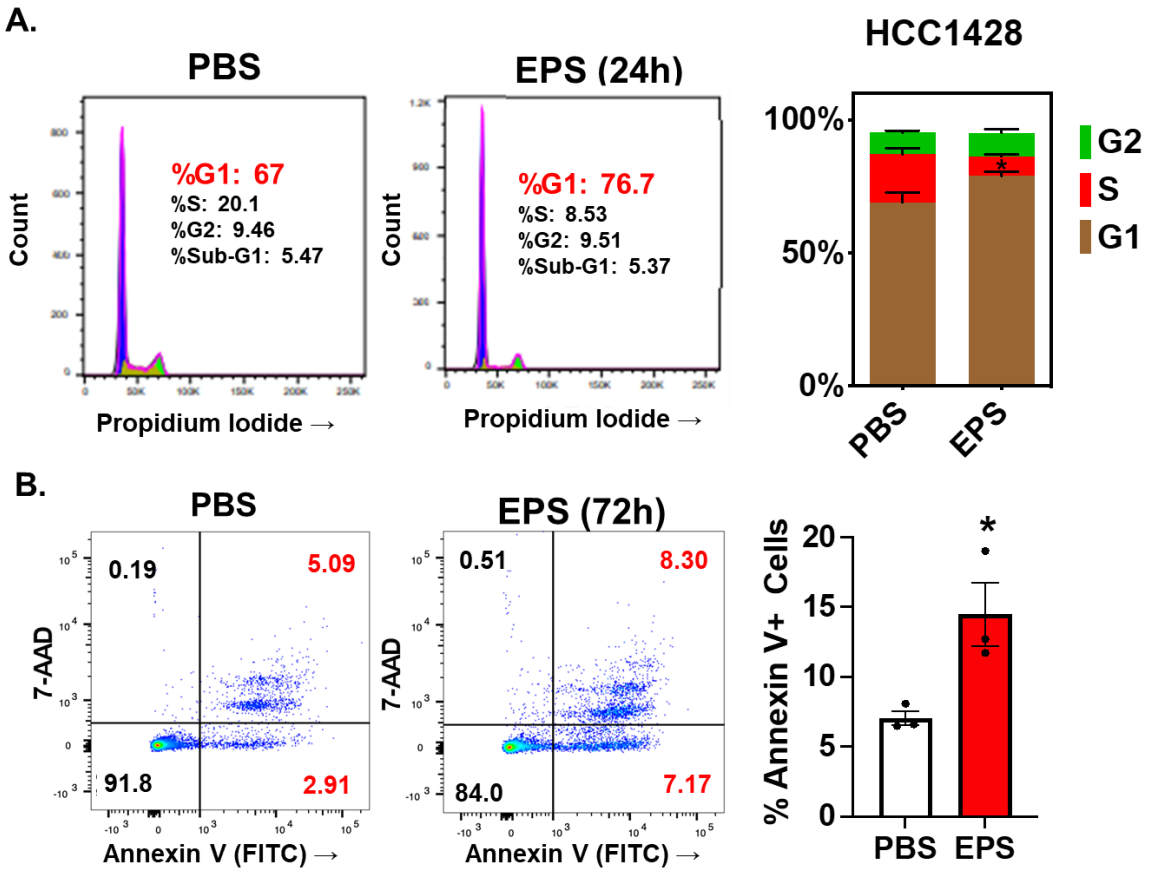


Figure 6. Effects of EPS on Cycle Cell and Cell Death in HCC1428 Cells. A. HCC1428 cells were treated with PBS or 5µg/mL EPS for 24h and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Representative flow plots (left) and graph (right): Data are represented as mean ± SEM of 3 independent experiments performed in triplicate, with t-test * $P \leq 0.05$. **B.** HCC1428 cells were plated in triplicate in 6-well plate and treated with PBS or 5µg/mL EPS for 3 days. Apoptosis was examined by staining with Annexin V and 7-AAD and analyzed by flow cytometry. Representative flow plots (left) and graph (right): Percent of Annexin V positive cells (including 7-AAD positive cells) as mean ± SEM of 3 independent experiments performed in duplicate, with t-test * $P \leq 0.05$.

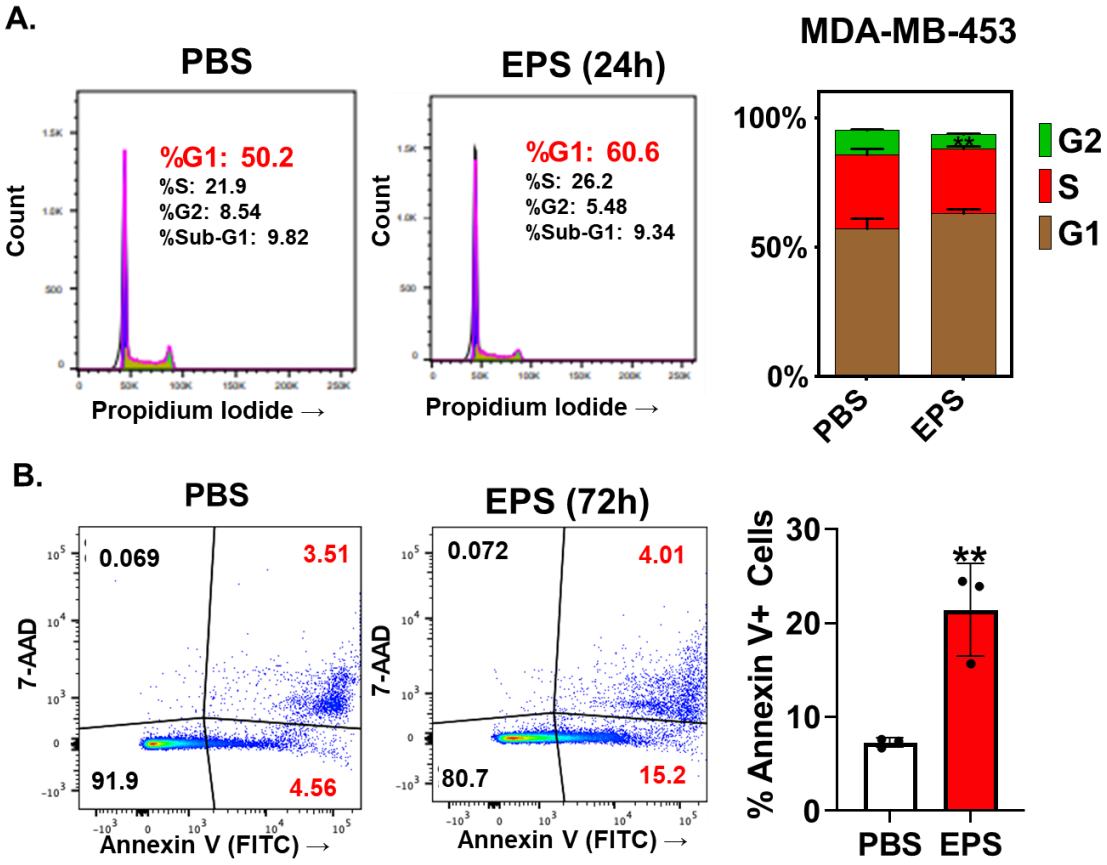


Figure 7. Effects of EPS on Cycle Cell and Cell Death in MDA-MB-453 Cells. A. MDA-MB-453 cells were treated with PBS or 5µg/mL EPS for 24h and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Representative flow plots (left) and graph (right): Data are represented as mean ± SEM of 3 independent experiments performed in triplicate, with t-test ** $P \leq 0.01$. **B.** MDA-MB-453 cells were plated in triplicate in 6-well plate and treated with PBS or 5µg/mL EPS for 3 days. Apoptosis was examined by staining with Annexin V and 7-AAD and analyzed by flow cytometry. Representative flow plots (left) and graph (right): Percent of Annexin V positive cells (including 7-AAD positive cells) as mean ± SD of only one experiment performed in triplicate, with t-test ** $P \leq 0.01$.

Inhibition of Metabolism of T47D Breast Cancer Cells

Metabolism is another important mechanism controlling cellular proliferation. The G1 checkpoint, in particular, considers metabolic cues for progression through the cell cycle (Lee & Finkel, 2013). EPS is a large molecule comprised of carbohydrates, which could potentially be metabolized by cancer cells leading to alterations of their normal glucose metabolism. Since EPS induces G1/G0 cell cycle arrest in T47D cells, we hypothesized that EPS could inhibit the metabolism of T47D cells leading to G1/G0 cell cycle arrest. To test this hypothesis, we measured metabolic capacities of T47D cells in the presence of EPS. T47D cells were either pretreated with EPS for 48h or acutely exposed to EPS for minutes before measurement of oxidative and glycolytic metabolisms using the Seahorse XF Cell Mito Stress Test Kit. If EPS inhibits T47D metabolism, we expect to see decreased oxidative and glycolytic metabolisms with the addition of EPS. We also expect longer treatment with EPS to have a more pronounced effect than acute treatment since EPS might take more time to alter metabolic gene expression. Pretreatment with EPS trended towards dampening both glycolytic and oxidative capacities in T47D cells (Figure 8). However, due to the small sample size (n=10) and the fact that the experiment was performed only once, the maximal respiration capacity of 48h EPS treatment was the only one reaching statistical significance (Figure 8B). Treatment of EPS for a few minutes showed a similar trend toward inhibiting metabolism, but the inhibition was not as pronounced as the prolonged treatment. These data suggest that EPS may need time to alter gene expression to inhibit metabolism instead of immediate interference of metabolic enzyme functions.

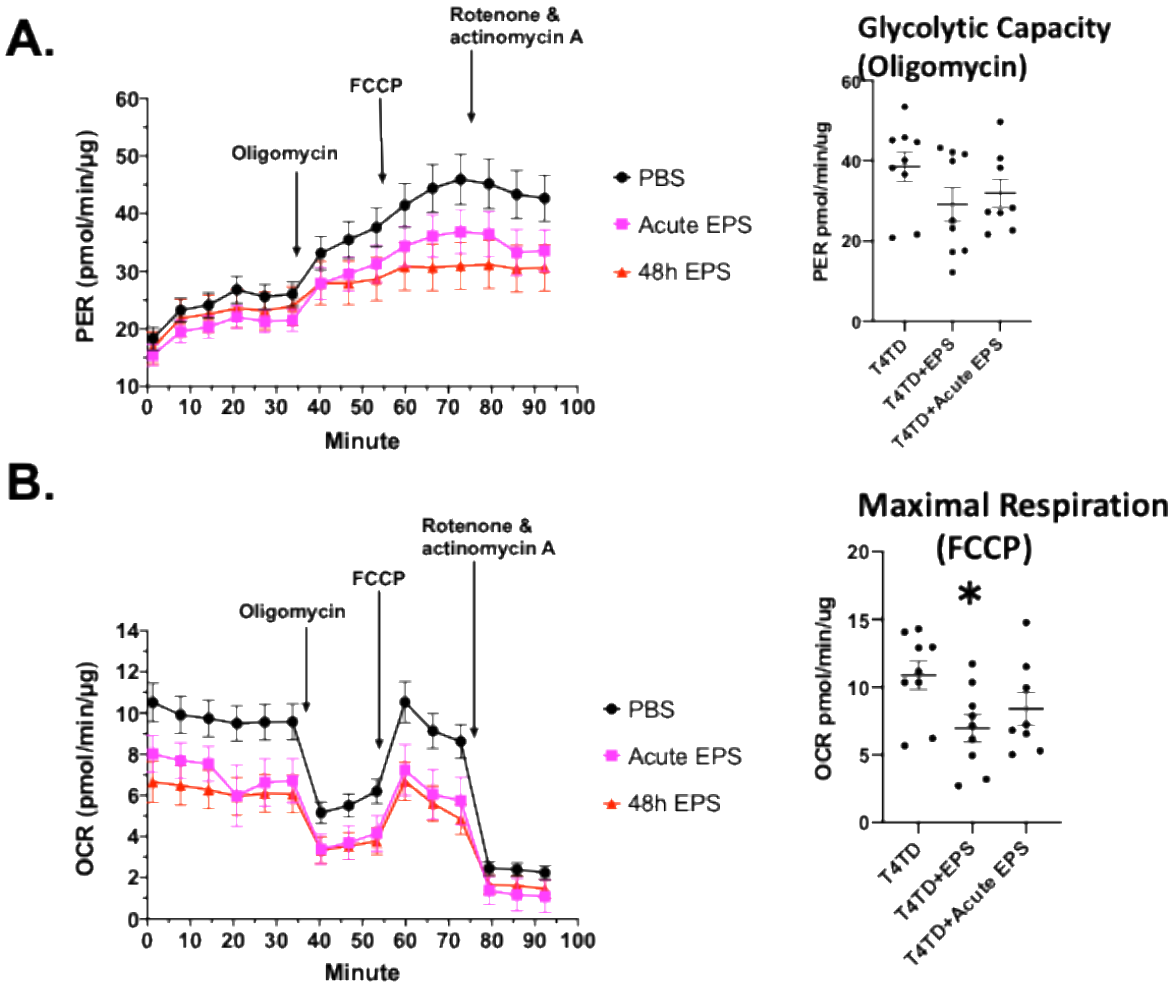


Figure 8. Effect of EPS Pretreatment on Glycolytic and Oxidative Metabolism of T47D Cells. Cells were plated at a density of 6000 cells per well into 96-well plate and pretreated with 5µg/mL EPS or PBS for 40 hours. Cells were then changed to seahorse media without serum for 2h in the normal incubator and then placed in incubator without CO2 for 1h before seahorse assay. For acute EPS treatment, EPS was injected into the PBS-pretreated wells immediately before the seahose assay. Seahorse XF Cell Mito Stress Test Kit were used to measure OCR and PER (Oligomycin = 1.5uM, FCCP = 0.5uM, 0.5uM Antimycin A + Rotenone) according to manufacturer's protocol. Metabolic rates were normalized by protein concentration in the well. **A.** PER glycolytic metabolism (left) with maximal capacity measured following Oligomycin addition (right) and **B.** OCR oxidative metabolism (left) with maximal capacity measured following FCCP addition (right) were graphed as mean ± SD (n=10 from 1 experiment), with t-test * P <0.05.

Reversibility of Short-term EPS Treatment

EPS inhibits proliferation of breast cancer cells. T47D cells, in particular, exhibit G1/G0 cell cycle arrest in the presence of EPS. We tested if effects of EPS on proliferation were reversible or irreversible by first treating with EPS and then measuring proliferation after removal of EPS. T47D cells were treated with EPS for 3 days and plated at equal densities to measure cell cycle progression and proliferation after EPS withdrawal. If EPS induces irreversible changes in T47D cells, we expect to see continued cell cycle arrest after EPS is removed. On the other hand, if EPS induces a temporary state of growth arrest, we expect EPS pre-treated cells to recover to their basal cell cycle progression 24 hours following removal of EPS compared to controls. Results supported the latter hypothesis in which removing EPS restored cell cycle progression within 24 hours (Figure 9A). Proliferation was also completely restored following EPS removal (Figure 9B). This finding suggests that the growth inhibition induced by EPS is reversible.

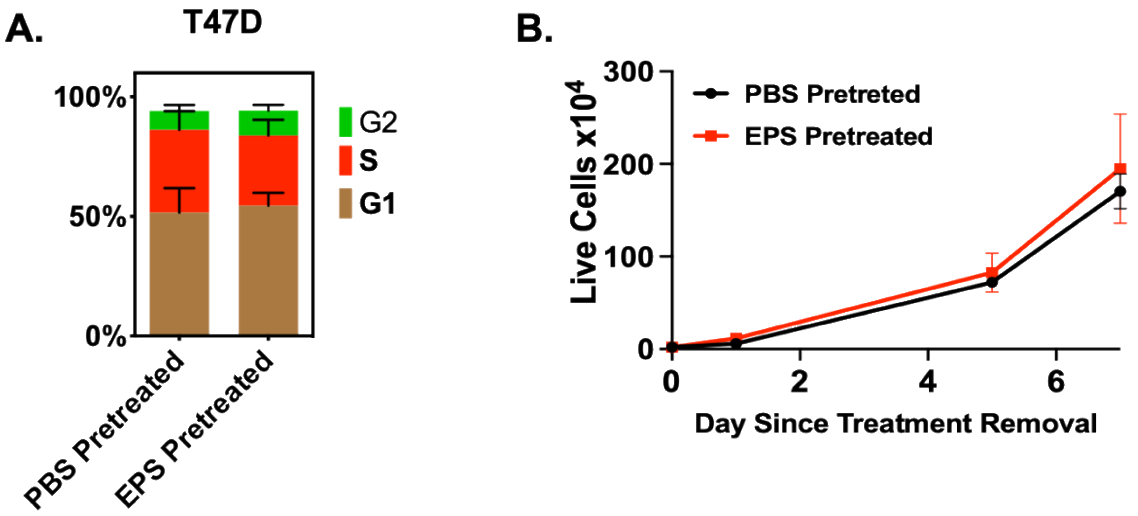


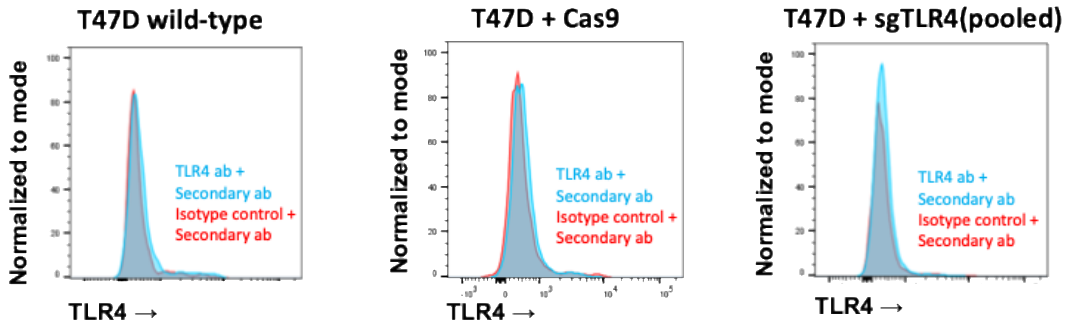
Figure 9. Cell Cycle Progression and Proliferation of T47D Cells Following Removal of EPS Treatment. T47D cells were pretreated with PBS or 5µg/mL EPS for 3 days, then cells were plated into a 12-well plate (20,000 cells/well) in regular media with treatment removed **A.** 24h following removal of treatment, cells were collected and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Data are represented as mean ± SD of 3 independent experiments performed as singlet, with the t-test not being significant. **B.** To measure proliferation, live cells were counted on day 1, 5 and 7 following removal of EPS, via trypan blue exclusion on a hemocytometer. Data are represented as mean ± SEM of 2 independent experiments each performed in triplicate, with the t-test not being significant.

Requirement of TLR4 in EPS-Mediated Inhibition of T47D Proliferation

It is well-established that EPS requires the toll-like-receptor 4 (TLR4) expression on myeloid cells to exert anti-inflammatory effects (Jones et al., 2014; Kalinina et al., 2021; Paik et al., 2020). Hence, we investigated whether TLR4 was also required for the growth inhibitory effect of EPS on breast cancer cells. We first examined whether T47D cells expressed TLR4 via flow cytometry and found that these cells had little detectible TLR4 (Figure 10A). Since the receptor could still signal with only a few receptors expressed, we knocked out (KO) TLR4 in T47D cells using CRISPR/Cas9. As we could not verify the knockout by protein expression, we obtained a single cell clone of the TLR4 knockout T47D cell lines in which both alleles displayed frameshift mutations where the sgRNA targeted that region of the gene (Figure 10B).

Then, we performed a proliferation assay with increasing concentrations of EPS using the TLR4 KO clone to determine whether these cells could respond to EPS. If EPS requires TLR4 to inhibit proliferation of T47D cells, then we expect that TLR4-KO cells will be insensitive to EPS and continue to proliferate. Results showed that the knockout of TLR4 did not prevent EPS-mediated inhibition of proliferation as compared to wild-type or Cas9-transfected control T47D cells (Figure 11). Thus, EPS does not require TLR4 to inhibit the proliferation of T47D cells, which suggests a distinct mechanism compared to immune cells.

A.



B.

Sample	DNA sequence
TLR4 Exon 1 (Ensembl)	CAGTGAGGATGATGCCAG**GATGATGTCTGCCTCGCGCC**TGGCTGGGACTCTGATCCCAGCCATGGCCTT
Cas9 only	CAGTGAGGATGATGCCAG**GATGATGTCTGCCTCGCGCC**TGGCTGGGACTCTGATCCCAGCCATGGCCTT
Cas9 & sgTLR4 Clone F6 allele 1	CAGTGAGGATGATGCCAG**GATGATGTCTGCCTCGC**C**GCC**TGGCTGGGACTCTGATCCCAGCCATGGCCTT
Cas9 & sgTLR4 Clone F6 allele 2	CAGTGAGGATGATGCCAG**GATGATGTCTGCC~~TCGCGCC~~~~TGGCTGGGACTCTGATCCC~~AGCCATGGCCTT

Figure 10. CRISPR Knockout of TLR4 in T47D Cells. A sgRNA was designed to knockout TLR4 in T47D cells, cloned into a Cas9-expressing vector and transfected into T47D cells. **A.** Following selection with purocymin, a pool of clones was stained with PE-TLR4 and analyzed via flow cytometry for expression of surface TLR4. B. Pooled cells were cloned, and a single clone of TLR4 KO (F6) cells was subjected to DNA sequencing of the TLR4 region targeted by the sgRNA (red font). Blue front depicted a single nucleotide insertion in an allele(1) of clone F6, while the strikethrough of red front depicted an 8-nucleotide deletion in allele(2).

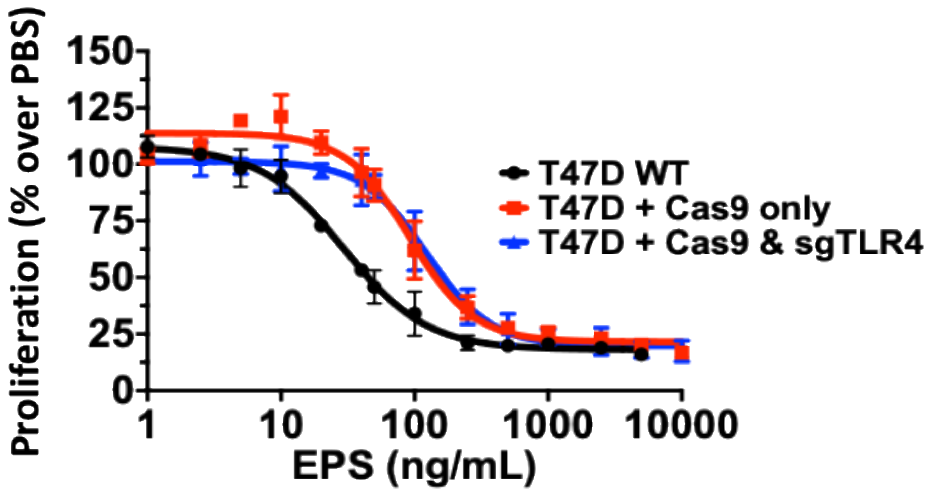


Figure 11. The Proliferation of TLR4 KO T47D Cells in Response to EPS. T47D cells (WT vs Cas9 vs TLR4 KO clone) were grown in media containing PBS or increasing concentration of *B. subtilis* EPS for 6 days. XTT absorbance assay was used to measure number of live cells in each treatment (n=6). The PBS-treated group was set at 100% proliferation. Percent proliferation of the EPS treated groups was calculated by dividing EPS over PBS multiplied by 100. The log(inhibitor) vs response – Variable slope (four parameters) model was fitted as shown to determine the IC50 (inhibitory concentration at 50%). Data are represented as mean ± SEM of 3 independent experiments.

Conclusions on Proliferation

EPS inhibits the proliferation of some breast cancer cell lines in a dose-dependent manner. EPS mainly induces cell death in HCC1428, MDA-MB-468, and MDA-453 cells. In contrast, EPS induces G1/G0 cell cycle arrest and slows the metabolism of T47D cells. This inhibition of T47D cells is reversible, as removal of EPS restores cell cycle progression within 24 hours. Unlike immune cells which require TLR4 to respond to EPS, T47D breast cancer cells do not require TLR4 for EPS to inhibit their proliferation.

Effects of EPS on Aggressive Cancer-Associated Phenotypes *in vitro*

Cell Migration

Besides proliferation, a thorough investigation of any new cancer agent should include assessment of other cancer-associated phenotypes, including cell migration, cancer stemness, and treatment resistance. These phenotypes are associated with more aggressive tumors. The ability of cancer cells to migrate is associated with cancer metastasis as it involves invasion through the basement membrane and migration to distant sites. Since EPS inhibits proliferation of some breast cancer cells, we hypothesized that EPS could also inhibit cancer cell migration. To test this hypothesis, the wound-healing scratch assay was performed on T47D cells to measure their migration capacity in response to EPS. T47D cells were plated into a 12-well plate and EPS was added for 48 hours until a confluent monolayer of cells was achieved. Two scratches crossing each other in the middle of the well were made using a pipette tip. Cells were incentivized to migrate closing the gap in low serum condition in the presence of PBS or EPS. Images of the same spots where the scratches crossed were taken at 10X magnification over 2 days to monitor gap closure. The rate of cell migration was estimated by how fast the gap closed over time. The rate of gap closure was measured qualitatively. If EPS inhibits cell migration, we expect EPS-treated T47D cells to close the gap at a slower rate than the control PBS-treated cells. Surprisingly, EPS treated T47D cells showed increased cell migration compared to PBS-treated cells (Figure 12). Experiments were repeated multiple times with similar results. Although EPS induced G0/G1 cell cycle arrest in T47D cells, it also increased their ability to migrate. This finding indicates that EPS may have multifaceted effects on breast cancer cells and may not be an anti-tumor agent.

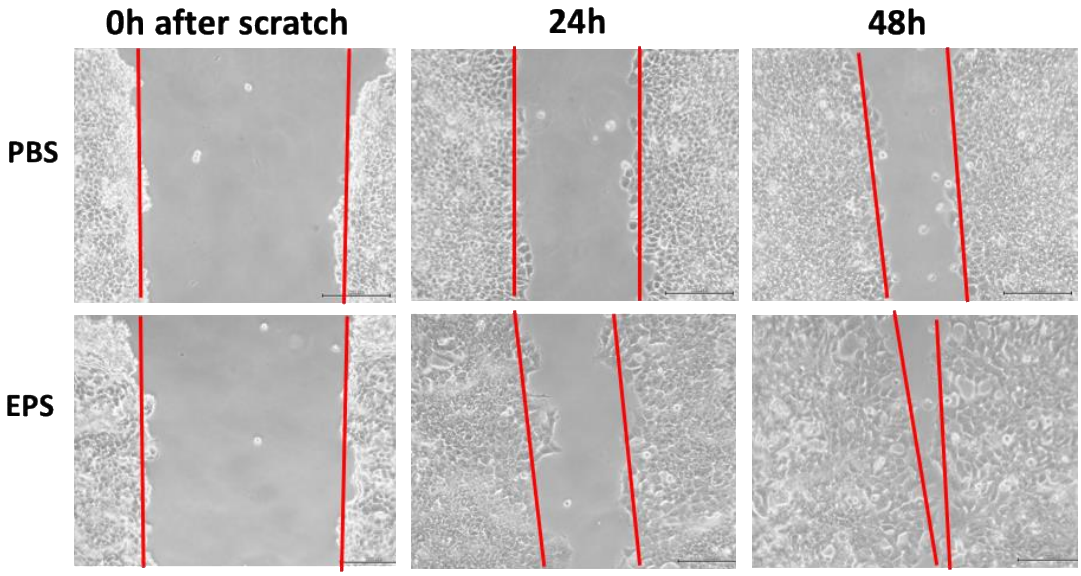


Figure 12. Effect of EPS on Migration of T47D Cells. T47D cells were pretreated with PBS or 5μg/mL EPS for 48h till a confluent monolayer of cells was achieved. Then cells were starved in 3% serum overnight. Cells were scratched using a pipette tip and open gaps were imaged at 10X magnifications. EPS treatment was maintained in 3% serum, and the same gaps were imaged again at 24h and 48h. Experiments were repeated >3 times, and representative images were shown.

Survival of Breast Cancer Stem Cells

Breast cancer stem cells (BCSCs), or tumor-initiating cells, is a small population of cells within bulk tumors displaying stem-cell properties. These cells are capable of self-renewal, differentiation along mammary epithelial lineages, extensive proliferation, and clonal nonadherent spherical clusters (mammosphere formation) (Fillmore & Kuperwasser, 2008; Ponti et al., 2005). Due to these stem-like characteristics, BCSCs are thought to be responsible for treatment resistance, recurrence and metastasis (Bartucci et al., 2015; Charafe-Jauffret et al., 2009; Creighton et al., 2009; W. Li et al., 2017; Palomeras et al., 2018; Rabinovich et al., 2018; Ricardo et al., 2011; Yin & Glass, 2011; Zhang et al., 2020). Thus, it is critical to assess the effect of a new cancer agent on survival of BCSCs. Although EPS-treated T47D cells are not

actively dividing, these cells migrate faster and may display more aggressive phenotypes. We hypothesized that EPS enriches for BCSCs of T47D cells, leading to a more aggressive cancer behavior. To test this hypothesis, we utilized the mammosphere formation assay, which is one method to assess the survival of BCSCs based on their ability to survive and proliferate in a 3D culture. Tumorspheres of cells within mammosphere medium larger than 50microns or 100microns are considered BCSC-enriched mammospheres. T47D cells were pretreated with EPS for 4 days before plating into mammosphere forming medium. Mammospheres were allowed to form for 7 days. If EPS enhanced BCSC survival, we expect to find more mammospheres in EPS-treated cells. Pretreatment with EPS increased mammospheres (>100microns) of T47D cells (Figure 13). Interestingly, when mammospheres that were greater than 50microns were counted, no difference was observed (see Figure 25 as these were part of the same experiment). These results suggest that EPS may be stimulating proliferation of BCSCs while inhibiting proliferation of the non-BCSCs.

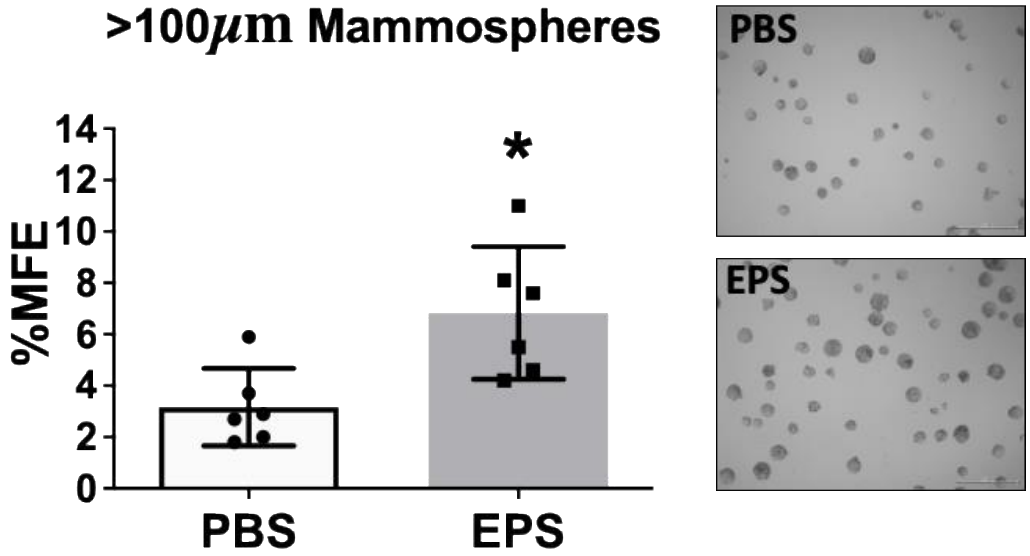


Figure 13. Effects of EPS on Survival of T47D Cancer Stem Cells. T47D cells were pretreated with PBS or 5µg/mL EPS for 4 days, then 25,000 single, live cells were plated into mammosphere forming media for 7 days. Representative images at 4X magnification are shown. Mammospheres larger than 100µm were counted to obtain percent mammosphere forming efficiency (%MFE = # Mammospheres/ 25,000 Cells Plated). Data are represented as mean ± SD of 6 independent experiments performed as a single replicate, with t-test * $P < 0.05$.

Resistance of T47D Breast Cancer Cells to Long-term EPS Treatment

Short-term treatment of T47D cells with EPS induced cell cycle arrest along with enhancement of aggressive cancer features including increased migration and enriched BCSCs. We showed that short-term effects of EPS are readily reversible upon removal of EPS. However, what is unknown is whether long-term treatment with EPS would induce resistance. As EPS is produced by a commensal bacterium which usually coexists long-term with the host, it is important to assess the response of cancer cells within the host to long-term EPS exposure. We hypothesized that long-term exposure to EPS would lead to resistance in T47D cells resulting in continued proliferation in the presence of EPS. To test this hypothesis, we cultured T47D cells for 60 days in the presence of PBS or 5µg/mL EPS and then performed cell cycle analysis and a proliferation assay to assess their response to EPS. As controls, cells cultured in PBS long-term

were tested for their ability to respond to short-term EPS treatment. We expected that long-term PBS-treated cells would growth arrest in response to short-term EPS. On the other hand, we expected long-term treatment with EPS would lead to resistance so that long-term EPS-treated cells would continue to proliferate. As expected, EPS induced G1/G0 cell cycle arrest and inhibited proliferation of the long-term PBS-treated control cells (Figure 14A, middle graph, Figure 14B). Compared to the long-term PBS-treated cells, the long-term EPS-treated cells exhibited minimal G0/G1 cell cycle arrest in response to EPS (Figure 14A, left graph). Similarly, proliferation of long-term EPS-treated cells (EPS 60D => EPS 6D) were nearly the same as long-term PBS-treated cells (PBS 60D => PBS 6D), indicating that EPS no longer inhibited the proliferation of these cells (Figure 14B). Removal of EPS from these long-term EPS-treated cells had no impact on the distribution of cell cycle phases (Figure 14A, right graph) or on their proliferation (Figure 14B). Together, these findings suggest that long-term exposure to EPS leads to resistance and altered response, where the presence of EPS no longer inhibited cell growth.

To identify a mechanism leading to resistance of cells treated with long-term EPS, we tested effects of EPS on survival of BCSCs using the mammopsheres formation assay. EPS increased the number of mammospheres compared to PBS (Figure 14C), suggesting that EPS potentially enriched BCSCs within the bulk cell population. In addition, removing EPS from these long-termed EPS-treated cells for a few days was sufficient to decrease mammosphere efficiency to PBS control levels (Figure 14C). This finding suggests that the effect of EPS on BCSCs is also reversible. EPS may maintain higher level of BCSCs as long as it is present to the cells. However, more studies are needed to further delineate how EPS enhances for BCSCs and how that plays into long-term resistance against growth inhibition.

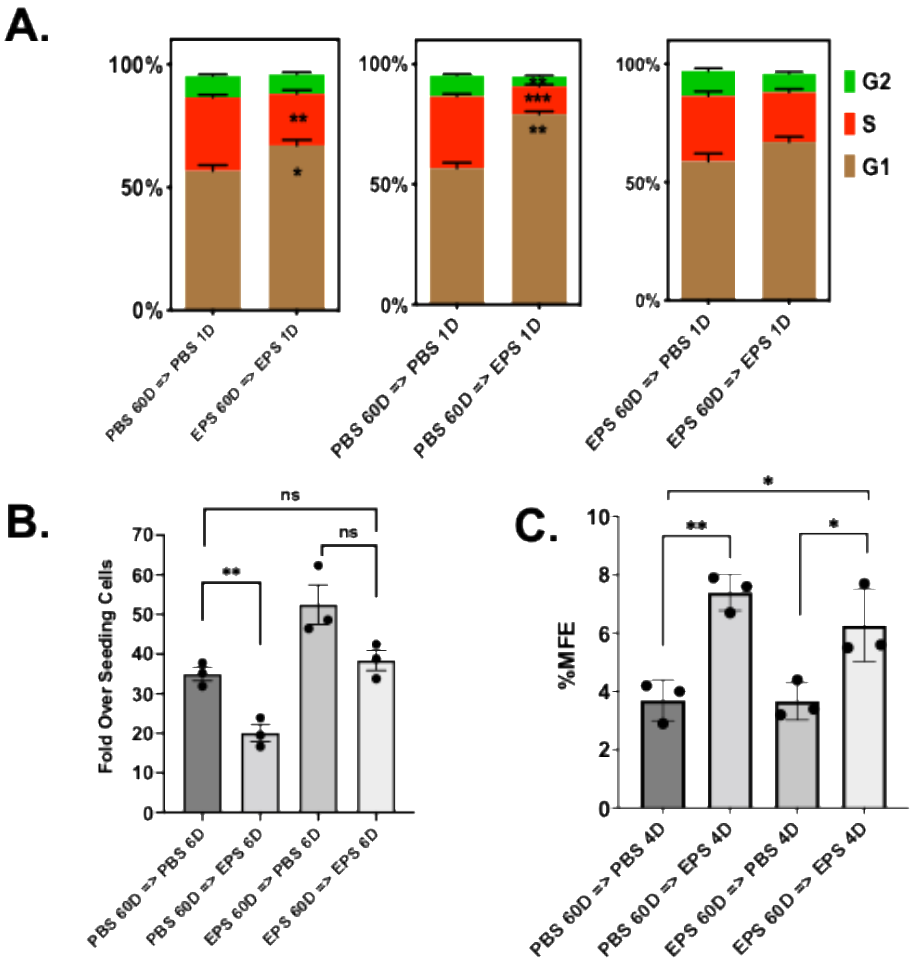


Figure 14. Effect of Long-term EPS Treatment on Cell Cycle Progression, Proliferation, and Mammosphere Formation of T47D Cells. T47D cells were cultured long-term (60 days) in media containing PBS or 5µg/mL EPS, denoted as PBS 60D or EPS 60D. **A.** Cell cycle analysis was performed on long-term cultured cells that was short-term treated with 5µg/mL EPS or PBS for 24h (denoted as PBS/EPS 60D => PBS/EPS 1D). Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Data are represented as mean ± SEM of 3 independent experiments performed in triplicate, with t-test * $P \leq 0.05$, ** $P \leq 0.01$, *** ≤ 0.001. **B.** Growth assay was performed on long-term cultured cells in the presence of 5µg/mL EPS or PBS (denoted as PBS/EPS 60D => PBS/EPS 6D). Live cells were counted on day 6 by trypan blue exclusion on a hemocytometer. Proliferation was calculated as Fold Over Seeding Cells = (# Live Cells on Day 6)/(# Live Cells Plated on Day 0). Data are represented as mean ± SEM of 3 independent experiments performed in triplicate, with t-test **$P \leq 0.01$. **C.** Long-term cultured cells were pretreated with 5µg/mL EPS or PBS for 4 days (denoted as PBS/EPS 60D => PBS/EPS 4D), then 25,000 single, live cells were plated into mammosphere forming media for 7 days. Mammospheres larger than 100µm were counted to obtain percent mammosphere forming efficiency (%MFE = # Mammospheres/ 25,000 Cells Plated). Data are represented as mean ± SD of 3 independent experiments performed in single replicate, with t-test * $P < 0.05$, ** $P \leq 0.01$.

Effect of EPS on the Growth of Breast Tumor Xenografts *in vivo*

Growth of EPS-Treated T47D Tumor Xenografts in Nude Mice

To determine the physiological role and implication of long term EPS treatment on breast tumor growth, we utilized an orthotopic xenograft model in which ER+ T47D human breast cancer cells are implanted as xenografts into athymic, nude mice. To ensure that cells fully exhibit EPS-induced phenotypes, we pretreated T47D cells *in vitro* with 5µg/mL EPS for 8 days. Then, 4 million cells suspended in Matrigel along with EPS were injected bilaterally into the fourth mammary fat pads of female, ovariectomized nude mice. Hormone-sensitive T47D tumors were allowed to develop in the presence of a silastic capsule that releases 83-120pg/mL 17β-estradiol over an 8-week period, after which the capsule was replaced. Mice from each group (4 per group) were continued to be treated with either PBS or 50µg EPS via intraperitoneal (i.p) injection thrice weekly. Tumor area was measured weekly and mice were euthanized on day 94 following injection. If EPS enhanced more aggressive tumor features with long-term treatment *in vivo*, we expected EPS would increase tumor area compared to PBS. We found that EPS treatment significantly increased the rate of tumor growth in nude mice (Figure 15A). EPS also trended towards increasing the mass of tumors (Figure 15B). Together, these *in vivo* findings indicate that EPS acts as a pro-tumorigenic agent rather than inhibiting tumor growth as with short-term treatment *in vitro*.

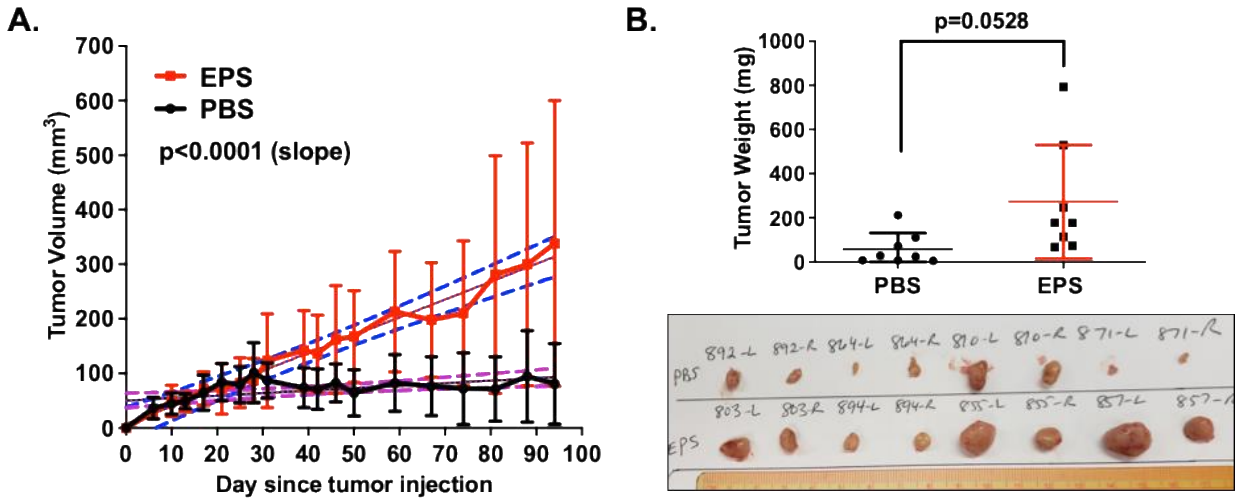


Figure 15. Effect of EPS Treatment on T47D Xenograft Tumors Growth in Nude Mice. T47D cells were pretreated with 5µg/mL EPS or PBS in vitro for 8 days, and $4x10^6$ cells were injected, bilaterally, into mammary fat pads of female, ovariectomized, foxn1 nu/nu, athymic nude mice. Each mouse was tagged on the ear with an identifiable number. All mice were implanted with a capsule releasing 83-120pg/mL 17β-estradiol, which was replaced at 8^{th} week. 4 mice each were implanted with EPS or PBS-pretreated cells and i.p injected with respective 50µg EPS or 100µl PBS 3 times/week. Tumor area (mm^2) was measured using Vernier calipers every week for 94 days. **A.** Graph shows mean ± SD of 8 tumors per group. Linear regression analysis was performed to calculate slopes of tumor growth for each treatment group. A Student's t- test was used to assess statistical significance between slopes. **B.** Mice were euthanized on day 94 and tumors were imaged and weighted. A Student's t-test was used to assess statistical significance between treatment groups.

Growth of EPS-Treated T47D Tumor Xenografts in NOD/SCID Mice

EPS can act directly on breast cancer cells to modulate their phenotypes. Additionally, EPS stimulated tumor growth in nude mice, suggesting that EPS directly acts on cancer cells to promote tumor growth. However, EPS has been shown to induce an anti-inflammatory state. Hence, it is possible that EPS may indirectly promote tumor growth by inducing a tolerogenic immune state. Although nude mice lack a functional thymus, they have a functional innate

immune compartment as well as extrathymic T cell development. As EPS is known to impact myeloid cells, we required a different immunocompromised mouse model to distinguish between the direct effects of EPS on cancer cells versus the indirect effects of EPS via its modulation of immune cells. Thus, we tested the effect of EPS on tumor growth using a more immunocompromised mouse model, NOD/SCID, that lacks innate immune function. The experiment was performed similarly as with the nude mice. Tumor area was measured weekly and mice were euthanized on day 87 following injection. If EPS acted directly on cancer cells to promote tumor growth, we expected that the EPS-treated mice would have larger tumors similar to what we found previously using nude mice. In contrast, if EPS required immune cells to indirectly promote tumor growth, we expected to see little difference in the growth of tumors from EPS-treated NOD/SCID mice compared to PBS-treated mice. Results support the former hypothesis. Similar to the T47D tumor xenograft study in nude mice, EPS treatment also increased rate of tumor growth and tumor mass in NOD/SCID mice, suggesting that EPS-mediated tumor promotion may directly affect cancer cells (Figure 16).

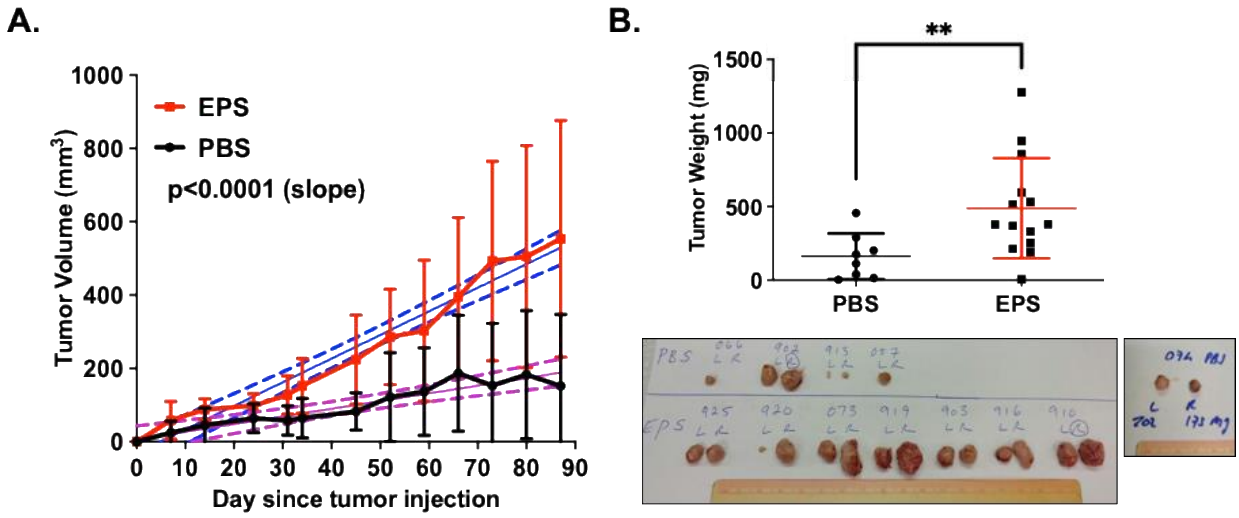


Figure 16. Effect of EPS Treatment on T47D Xenograft Tumors Growth in NOD/SCID Mice. T47D cells were pretreated with 5µg/mL EPS or PBS *in vitro* for 8 days, and $4x10^6$ cells were injected, bilaterally, into mammary fat pads of female, ovariectomized, NOD/SCID mice. Each mouse was tagged on the ear with an identifiable number. All mice were implanted with an estrogen capsule which releases 83-120pg/mL 17β-estradiol over an 8- week period. 4-7 mice each were implanted with EPS or PBS-pretreated cells and i.p injected with respective 25µg EPS or 100µl PBS 3 times/week. Tumor area (mm^2) was measured using Vernier calipers every week for 87 days. **A.** Graph shows mean ± SD of 8-14 tumors per group. Linear regression analysis was performed to calculate slope of tumor growth for each treatment group. A Student's t-test was used to assess statistical significance between slopes. **B.** Mice were euthanized on day 87 and tumors were imaged and weighted. A Student's t-test was used to assess statistical significance between treatment groups, ** $P \leq 0.01$.

Conclusions on the Pro-Tumorigenic Effects of EPS

Although short-term treatment of EPS *in vitro* leads to cell cycle arrest and temporary growth inhibition of T47D cells, EPS overall acts more like a tumor-promoting agent. Removal of EPS quickly restores cell cycle and cell growth. Moreover, EPS enhances phenotypes associated with aggressive tumors, including increased cell migration and BCSC survival. Long-term exposure to EPS enhances the aggressive phenotypes as these cells are no longer inhibited in the presence of EPS. They also maintain higher level of BCSCs compared to controls, which may explain their resistant behavior. Long-term treatment of EPS in T47D xenograft models in nude mice and NOD/SCID mice likewise leads to faster tumor growth and larger tumor mass.

These findings suggest that the tumor-promoting effects of EPS are potentially independent of immune cells, likely through novel direct interactions between EPS and breast cancer cells.

Mechanism by which EPS Alters Breast Cancer-Associated Phenotypes

Identification of Top Signaling Pathways Altered by EPS via RNA-SEQ

We employed an unbiased approach to discover mechanisms by which EPS modulates phenotypes of breast cancer cells. We hypothesized that EPS alters pathways only in the sensitive but not resistant cell lines to induce the associated phenotypes. Thus, we aimed to identify genes and pathways altered by EPS only in sensitive cells and not in resistant cells. RNA-sequencing (RNA-SEQ) was performed in two sensitive cell lines (T47D and MDA-MB-468) and two resistant cell lines (MCF-7 and MDA-MB-231) treated with EPS for 20 hours. Pathway analysis of the RNA-SEQ data showed that the top pathways altered in EPS treated T47D cells were DNA replication and G1 transition, in agreement with the G1 cell cycle arrest induced by EPS (Figure 17). In addition, pathways related to bacterial/viral infection and immune responses were also among the top pathways altered by EPS, including the interferon and TNF signaling (Figure 17). We hypothesized that EPS activates critical pathways leading to the observed phenotypes and identified 290 genes that were upregulated by EPS only in the sensitive but not resistant cell lines. Gene enrichment analysis was performed on this set of genes using Metascape pathway analysis software. The canonical NF-κB was identified as the top transcription regulators of these genes (Figure 18). Together, these data suggest that these breast cancer cells are sensing/responding to a bacterial molecule and activating the inflammatory response. Then, we focused on interrogating these pathways (TNF, Interferon/JAK-STAT and NF-κB) as potential mediators of EPS to induce cancer-associated phenotypes.

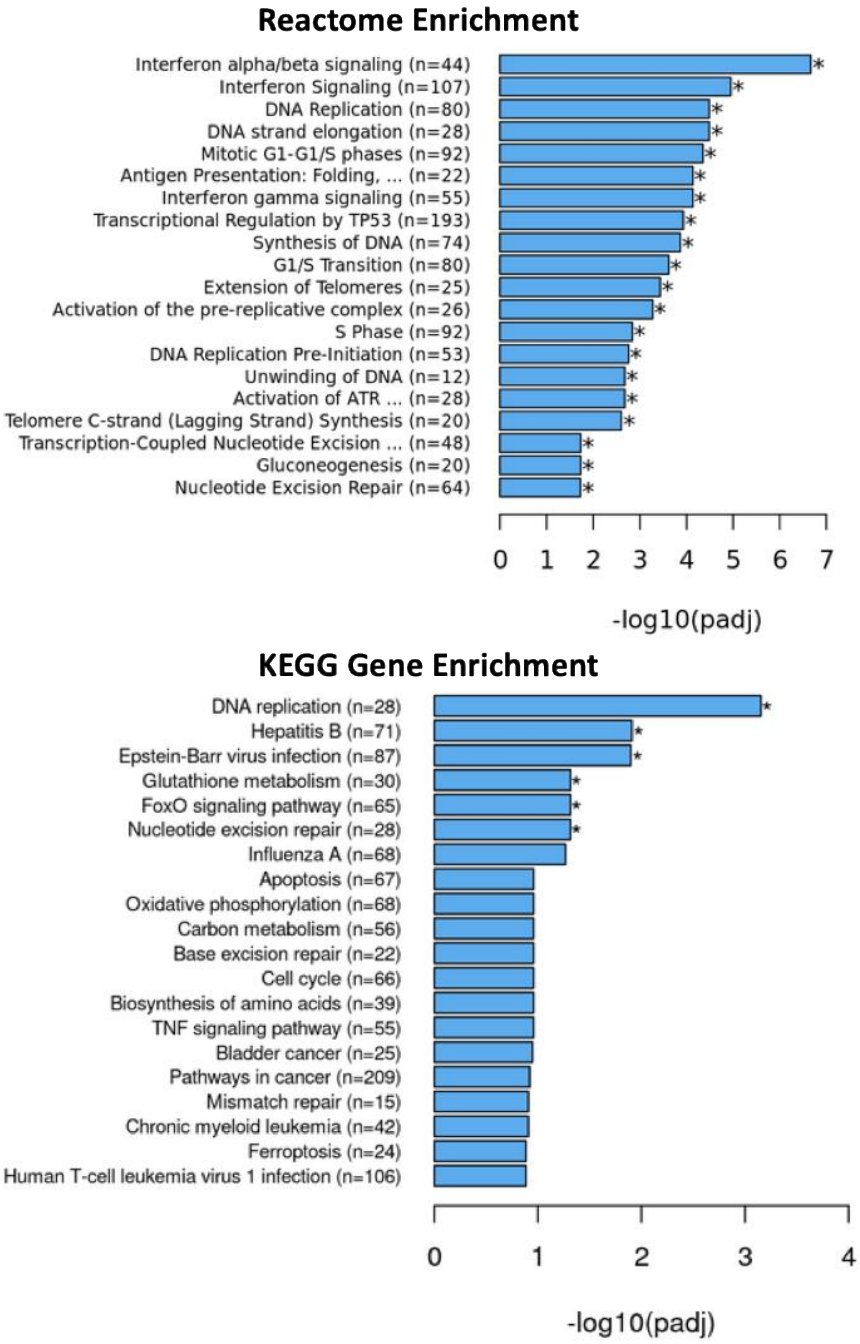


Figure 17. Top Pathways Altered by 20h-EPS Treatment in T47D Cells. EPS-sensitive T47D cells were treated with 5µg/mL EPS or PBS for 20h, and RNA-SEQ was performed. KEGG and Reactome gene enrichment pathways were analyzed. The data represent the log10 p-values on the X-axis and gene enrichment pathways on the Y-axis. The p-values were calculated based on triplicate samples.

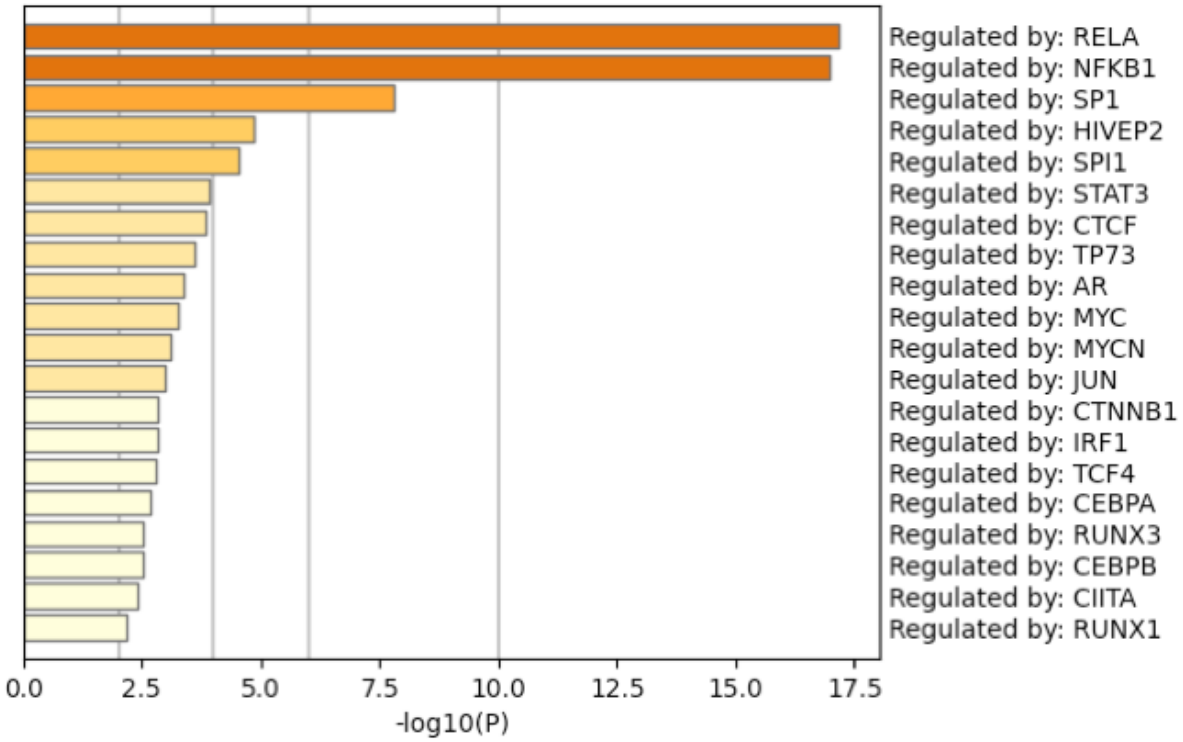


Figure 18. Top Transcription Regulators of Genes Upregulated by EPS in Sensitive Breast Cancer Cell Lines. Two sensitive (T47D and MDA-MB-468) and resistant (MCF-7 and MDA-MB-231) cell lines were treated with 5µg/mL EPS or PBS for 20h. RNA was collected and sequenced. Data are based on three biological replicates. 290 genes were identified as being upregulated by EPS only in the sensitive but not resistant cell lines. Gene enrichment analysis was performed in GO_TRRUST on these genes using Metascape. The top transcription regulators are shown along with calculated p-values.

Activation of STAT1, NF-κB and Other Signaling Pathways by EPS

As molecules tend to bind their receptor and activate signaling pathways leading to downstream effects, we hypothesized that EPS potentially activates a signaling pathway(s) to mediate its phenotype on breast cancer cells. Based on the RNA-SEQ data, the most likely candidates are the top pathways relating to inflammatory response induced by EPS, including the Interferon/JAK-STAT and NF-κB pathways. We first asked whether these pathways are activated by EPS. We used western blot analysis to examine protein expression suggestive of pathway activation. T47D cells were treated with EPS over time (0, 5min, 20min, 1.7h, 3.3h,

24h) and lysates were prepared. Using specific phosphorylation as activation markers, we confirmed the activation of STAT1 and canonical NF-κB/P65 by EPS (Figure 19). We also observed weaker activation of STAT3, P38/MAPK, and noncanonical NF-κB/RelB (Figure 19 and Figure 20). In terms of timing, the activation of canonical NF-κB, as indicated by phosphorylation of P65, occurred within minutes of EPS treatment (Figure 19 and Figure 20). Activation of STAT1, as measured by phosphorylation of the tyrosine 701 residue, required at least 2-3hrs (Figure 19). STAT1 and canonical NF-κB/P65 were also activated in other EPS sensitive cells, and not in the EPS resistant MCF-7 cells (Figure 21). These data suggest that activation of these pathways are shared across the different EPS sensitive cell lines, and may correlate with function since they are not activated in EPS resistant cell lines.

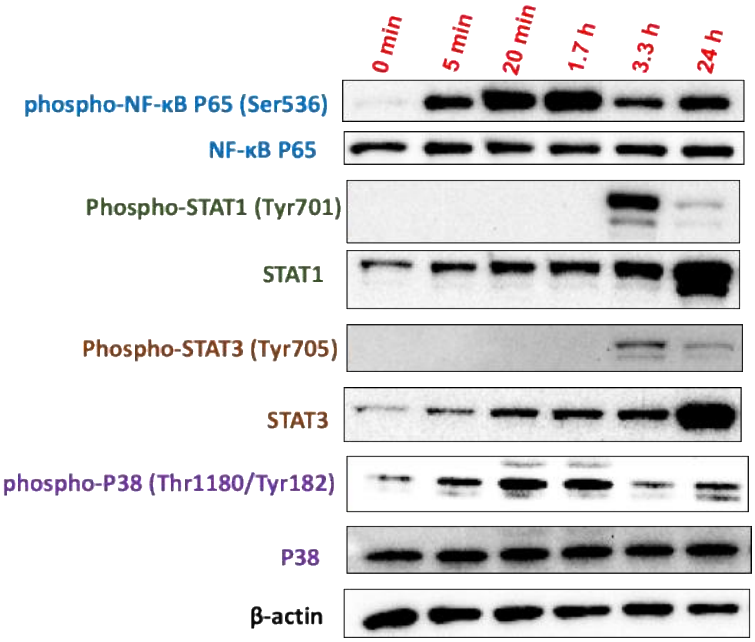


Figure 19. Western Blot Analysis of Signaling Pathways Activated by EPS in a Time Dependent Manner. EPS-sensitive T47D cells were treated with 5µg/mL EPS for 0 min, 5min, 20min, 100 min (1.7hrs), 200min (3.3hrs), and 24hrs. Total cell lysates were collected and proteins detected by western blotting with antibodies against indicated proteins. ß-actin was used as a loading control. The experiment was repeated at least 2 independent times. A representative image for each protein is shown.

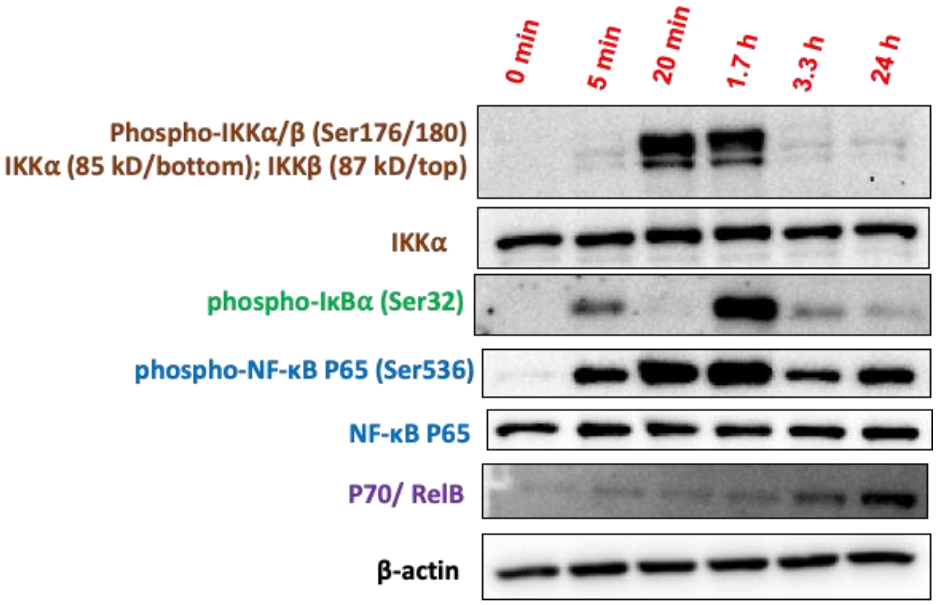


Figure 20. Western Blot Analysis of Canonical and Noncanonical NF-κB Pathways Activated by EPS in T47D Cells. EPS-sensitive T47D cells were treated with 5µg/mL EPS for 0 min, 5min, 20min, 100 min (1.7h), 200min (3.3h), and 24h. Total cell lysates were collected, and proteins of interest were detected by western blot using antibodies against indicated proteins. ß- actin was used as a loading control. Experiments were repeated at least 2 independent times. A representative image for each protein is shown.

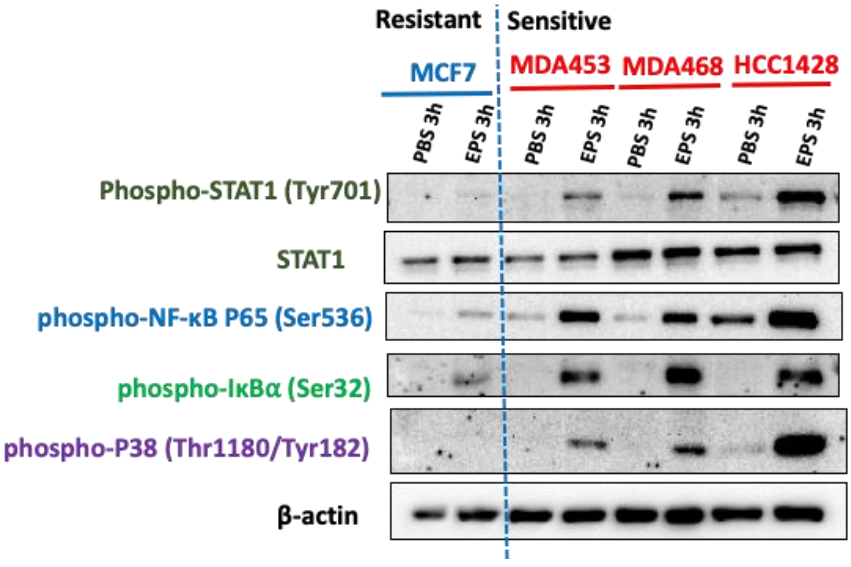


Figure 21. Pathways Activated by EPS in Sensitive vs Resistant Breast Cancer Cells. EPS-sensitive and resistant cells were treated with 5µg/mL EPS for 3h. Total cell lysates were collected and proteins of interest were detected by western blots with antibodies against indicated proteins. ß-actin was used as a loading control. Experiments were repeated twice. A representative image for each protein is shown.

Induction of TNFα and its Effect on Proliferation of EPS-Sensitive Cell Lines

EPS was shown to induce secretion of IL10 and TGF-β from myeloid cells to mediate immunosuppression (Kalinina et al., 2021; Paynich et al., 2017). Hence, we explored the possibility that EPS may induce secretion of cytokines to mediate the growth inhibition of breast cancer cells. TNFα was one of the top signaling pathways identified by RNA-SEQ analysis of genes induced by EPS in T47D cells (Figure 17) and MDA-MB-468 cells (Data not shown). Analysis of RNA-SEQ data further revealed that TNFα was only induced by EPS in the sensitive and not resistant cells (Figure 22A). To verify the induction of the TNFα protein, 4 breast cancer cell lines were treated with PBS or EPS for 2.5 hours and media was collected for cytokine-bead array (CBA) to measure levels of cytokines IL5, IL13, IL2, Il6, IL9, IL10, IL17a, IL17f, IL4, IL22, TNFα and IFNɣ. We confirmed that EPS increased secretion of TNFα at 2.5 hours from all 3 sensitive cell lines but not in the resistant MCF-7 cells (Figure 22B). There was no measurable secretion of other cytokines observed in the CBA, except for IL6 (Data not shown). In terms of timing of induction, we found that EPS treatment of T47D cells induced TNFα mRNA as soon as 40mins (Figure 23A), and protein at 2.5 hours which decreased at 22.5 hours (Figure 23B). Moreover, treatment of T47D cells with TNFα showed a similar phenotype to EPS treatment in inducing G1 cell cycle arrest (Figure 24A) and inhibition of proliferation (Figure 24B). Taken together, TNFα appeared promising as an effector molecule induced by EPS in sensitive cells to mediate growth inhibition.

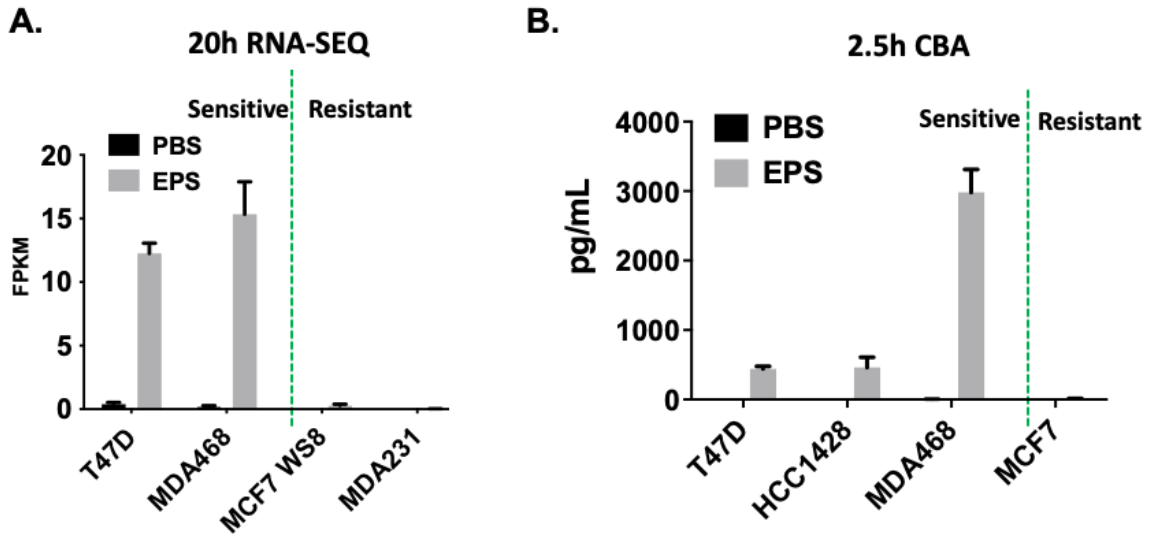


Figure 22. Induction of TNFα by EPS in Sensitive vs Resistant Breast Cancer Cells. A. RNA-SEQ analysis showing absolute TNFα gene expression in two sensitive (T47D and MDA-MB-468) and resistant (MCF-7 and MDA-MB-231) cell lines treated with 5µg/mL EPS or PBS for 20h. FPKM (absolute RNA expression) data are shown as mean ± SD for 3 biological replicates. **B.** Cells were treated with 5µg/mL PBS or EPS for 2.5h. Media were collected and cytokine bead array (CBA) analysis was performed to quantify inflammatory cytokines secreted into the media. Analysis was performed with Flow Jo. Data are shown as mean ± SD of one experiment performed in duplicate.

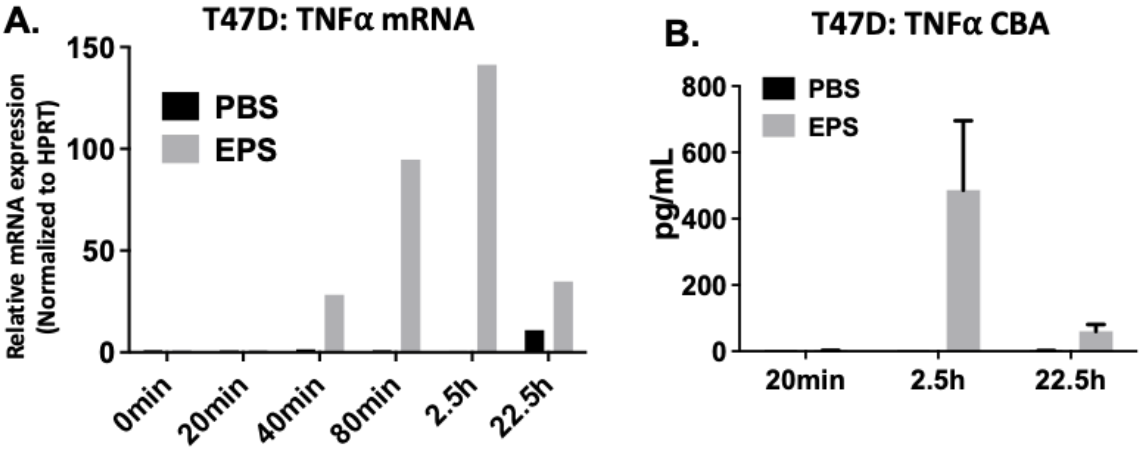


Figure 23. Time of TNFα Induction by EPS in T47D Cells. A. T47D Cells were treated with 5µg/mL PBS or EPS for varying amount of time before RNA was collected. qPCR was performed for TNFα and gene expression was normalized to HPRT and relative to PBS control using the $2^{-\Delta\Delta Ct}$ calculation in one experiment. **B.** 10,000 cells were treated with 5µg/mL PBS or EPS for 20min, 2.5h, or 22.5h before media collection. Cytokine bead array analysis was performed via flow cytometry to determine the amount of a number of inflammatory cytokines secreted. Analysis was performed with FlowJo. Data are shown as mean ± SEM for 2 independent experiments each performed in duplicates.

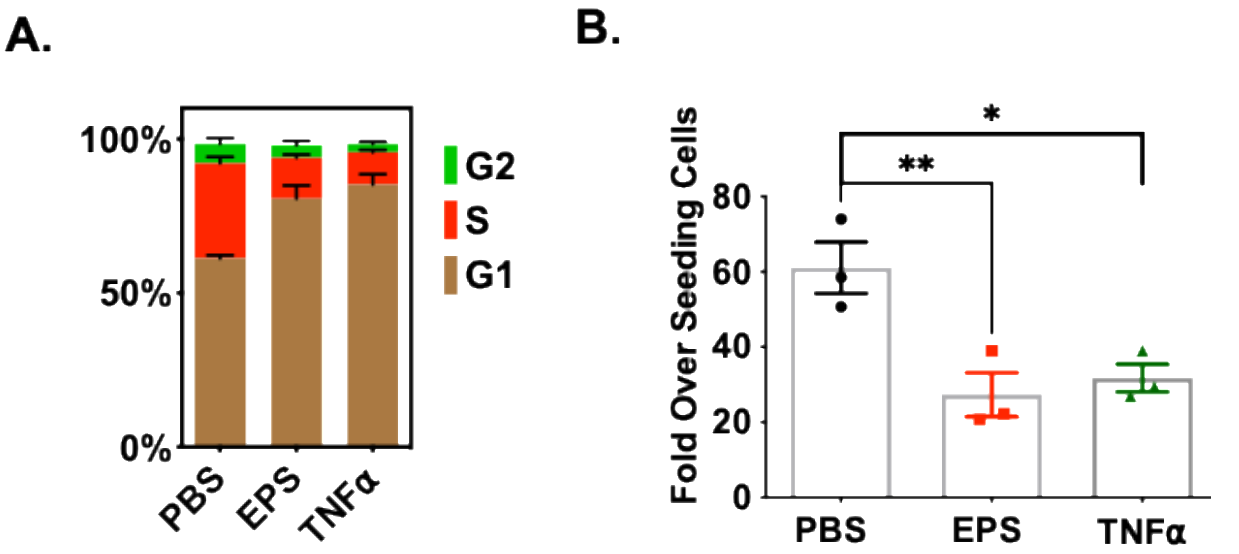


Figure 24. Effect of TNFα on T47D Cell Cycle Progression and Proliferation. A. T47D cells were plated in 12-well plates and treated with 5µg/mL EPS or 10ng/mL TNFα for 24h. Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Data are represented as mean ± SEM of 2 independent experiments performed in triplicate. **B.** Growth assay was performed in the presence of 5µg/mL EPS or 10ng/mL TNFα for 6 day. Live cells were counted by trypan blue exclusion on a hemocytometer. Proliferation was calculated as Fold over seeding cells = (# Live Cells on Day 6)/(# Live Cells Plated on Day 0). Data are represented as mean ± SEM of 3 independent experiments each performed in triplicate, with t-test * $P \leq 0.05$, ** $P \leq 0.01$.

EPS induced TNFα secretion only in sensitive cells and not resistant cells. Adding TNFα to the medium recapitulated the G1/G0 cell cycle arrest induced by EPS. Hence, we hypothesized that EPS induced G1/G0 cell cycle arrest in T47D cells by increasing TNFα. To test whether TNFα signaling is required for EPS-mediated inhibition of proliferation and induction of cell cycle arrest, we blocked TNFα using neutralizing antibodies to TNFα and the TNFR1 receptor expressed in T47D cells. TNFα treatment was used as a positive control to verify that the blockade was effective. We pretreated T47D cells with TNF-blocking antibodies for 30mins followed by treatment with either PBS, EPS, TNFα, or both for 24 hr. If TNFα is required by EPS to induce cell cycle arrest, we expect EPS to fail to induce G1/G0 cell cycle arrest in the

presence of TNFα/TNFR1 neutralizing antibodies. Results showed that blockade of TNFα using antibodies was sufficient to block TNFα from inducing G1 cell cycle arrest (Figure 25A). However, it could not block EPS from inducing cell cycle arrest in these cells (Figure 25A), suggesting that other pathways may be responsible for this phenotype. We also confirmed that TNFα blockade was insufficient to block signaling pathways induced by EPS, including phospho-STAT1, phospho-P65 and phospho-P38 (Figure 25B). In conclusion, TNFα signaling was not necessary for EPS-induced cell cycle arrest of breast cancer cells.

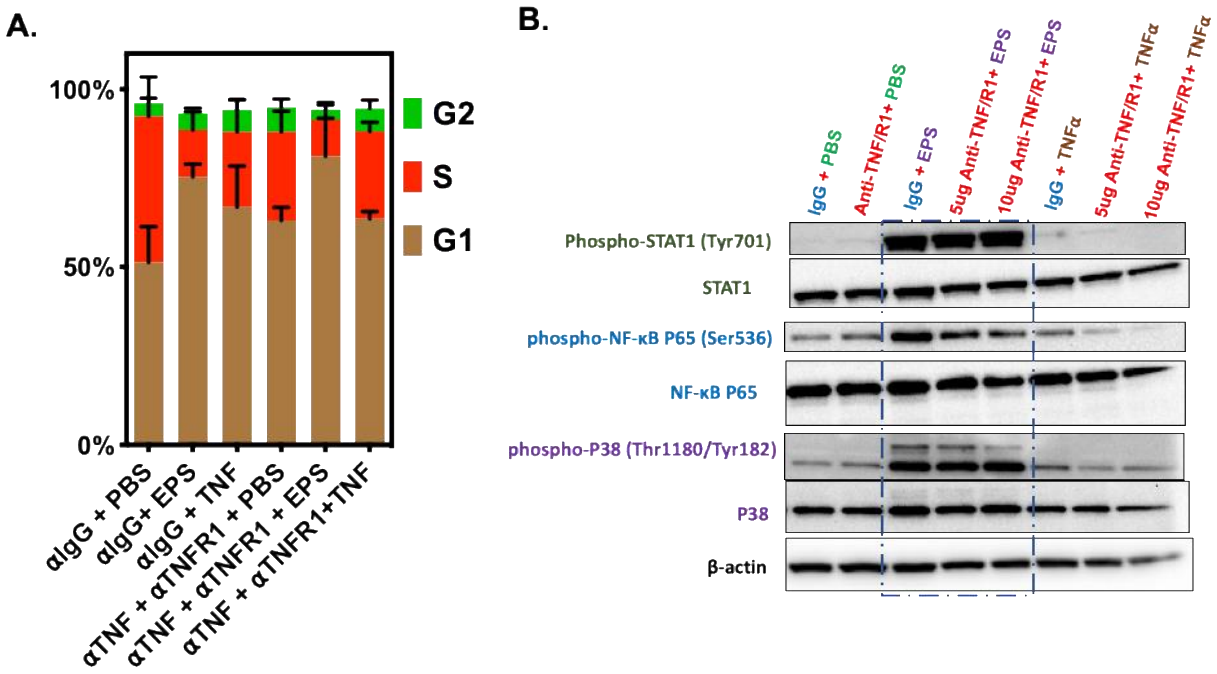


Figure 25. Effect of TNFα Blockade Using Blocking Antibodies on EPS-induced G1 Cell Cycle Arrest and Pathway Activation of T47D Cells. A. T47D cells were pretreated with 5µg/mL of either IgG or antibodies blocking TNFα and TNFR1 for 30mins. Then 5µg/mL of EPS or 10ng/mL TNFα was added for 24h. Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Data are represented as mean ± SD of 2 independent experiments performed as singlet. B. T47D cells were pretreated with 5µg/mL of either IgG or antibodies blocking TNFα and TNFR1 for 30mins. Then 5µg/mL of EPS or 10ng/mL TNFα was added for 2h. Total cell lysates were collected and analyzed by western blotting with antibodies against indicated proteins. ß-actin was used as a loading control. Experiments were done twice. A representative image for each protein is shown

Inhibition of the NF-κB and STAT1 Pathways using the IKK Inhibitor TPCA-1

We initially hypothesized that EPS activates a signaling pathway(s) to induce cancer-associated phenotypes and confirmed that EPS activates NF-κB and STAT1, the two top pathways identified from the RNA-SEQ analysis. Then, we wanted to determine whether these pathways are required by EPS to induce some or all of its phenotypes in breast cancer cells, including cell cycle arrest, growth inhibition, cell migration, and mammosphere formation. Our rationale is that if we completely inhibit the correct pathway(s) required by EPS, then EPS will not be able to induce these phenotypes. We first targeted the NF-κB pathway since it was identified as the top regulator of genes induced by EPS in sensitive cells (Figure 18). We chose the chemical inhibitor TPCA-1 to target the NF-κB pathway. TPCA-1 is a potent inhibitor of IκB kinases (IKKs), displaying 22-fold selectivity for IKKβ over IKKα with an IC50 of 17.9 nM (Podolin et al., 2005). Although well-known as an NF-κB inhibitor, TPCA-1 is also an effective inhibitor of STAT3 (Nan et al., 2014). We first treated T47D cells with increasing concentration of TPCA-1 in the presence of PBS or EPS for 2 hours to determine whether TPCA-1 inhibited the EPS-mediated activation of NF-κB. Western blot analysis showed that TPCA-1 reduced phosphorylation of IκBα and p65 in a concentration-dependent manner (Figure 26A). Intriguingly, TPCA-1 increased phosphorylation of IKKα/β also in a concentration-dependent manner as it inhibits activation of downstream the NF-κB pathway (Figure 26A). It is poorly understood how TPCA-1 mechanistically inhibits IKKs. We thought that TPCA-1 could inhibit the function of IKKs, but that somehow leads to the accumulation of the phosphorylated IKK protein. Another unexpected finding is that TPCA-1, which has no known interaction with STAT1, decreases EPS- induced STAT1 phosphorylation in a

concentration-dependent manner (Figure 26B). TPCA-1 at 0.5μM or 1μM almost completely abrogated STAT1 phosphorylation while having little effect on p38 phosphorylation, suggesting that the effect could be specific (Figure 26B). In fact, TPCA-1 abrogates the phosphorylation of STAT1 in a much more dramatic manner than it did to p65. Although we intended to inhibit only the NF-κB pathway using the selective IKK inhibitor TPCA-1, it incidentally also abrogates EPS-induced activation of STAT1.

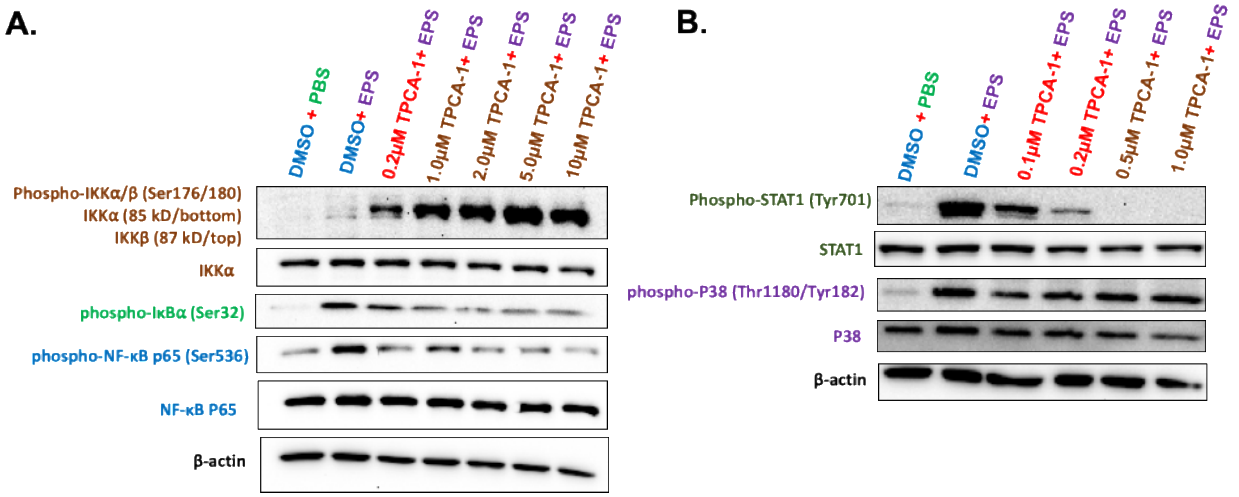


Figure 26. Effects of the IKK Inhibitor (TPCA-1) on EPS-Induced STAT1, NF-κB and P38 Signaling in T47D Cells. T47D cells were pretreated with DMSO or increasing concentrations of TPCA-1 for 30min, then 5μg/mL EPS or PBS was added for another 2h. Total cell lysates were collected and protein was detected by western blots using antibodies against indicated proteins. **A.** Expression of P-STAT1, total STAT1, P-P38, total P38 and loading control ß-actin proteins were in response to DMSO/PBS, DMSO/EPS, EPS plus 0.1mM, 0.2mM, 0.5mM, or 1.0mM TPCA-1. **B.** Expression of P-IKKa/ß, total IKKa, P-IKBa, P-P65, total P65, and loading control ß-actin proteins were detected with DMSO/PBS, DMSO/EPS, EPS plus 0.2mM, 1.0mM, 2.0mM, 5.0mM, or 10mM TPCA-1 treatment. Experiments were repeated 3 times. A representative image for each protein is shown.

We hypothesized that EPS requires NF-κB and/or STAT1 to induce the cancer-associated phenotypes in T47D cells: G1/G0 cell cycle arrest, growth inhibition, enhanced cell migrations and BCSC survival. Since TPCA-1 prevented EPS-mediated activation of both NF-κB and STAT1, we expected that TPCA-1 would reverse phenotypes induced by EPS. We first

tested the effect of TPCA-1 on EPS-induced cell cycle arrest. TPCA-1, at the concentration that it inhibited EPS-induced STAT1 phosphorylation (1μM), nearly completely rescued the G1/G0 cell cycle arrest induced by EPS in T47D cells. (Figure 27A). Then, we tested the effect of TPCA-1 on proliferation in response to PBS or EPS. TPCA-1 was not toxic to T47D cells at the concentration used to inhibit STAT1 (Figure 27B). TPCA-1 rescued EPS-mediated inhibition of proliferation in a dose-dependent manner (Figure 27B). Similarly, we performed a migration scratch assay in the presence of TPCA-1 and EPS. Here the wounds were quantified using ImageJ and percent wound closure was calculated. Pretreatment with 1μM TPCA-1 almost completely abrogated EPS-induced migration (Figure 28). Finally, we pretreated T47D cells with TPCA-1 and EPS before plating them into mammosphere medium. Results showed that while EPS alone increased the number of large mammospheres (>100μm) compared to PBS, TPCA-1 prevented this increase by EPS (Figure 29).

Overall, the IKK inhibitor, TPCA-1 rescued EPS- mediated decrease in proliferation, increase in cell cycle arrest, migration and survival of BCSCs of T47D cells. TPCA-1 was very efficient at rescuing all the phenotypes induced by EPS in T47D cells. However, its mechanism of action is complicated by the fact that it inhibits the activation of both NF-κB and STAT1 induced by EPS. Thus, more work is needed to delineate which of these two pathways is required by EPS, and for which phenotype.

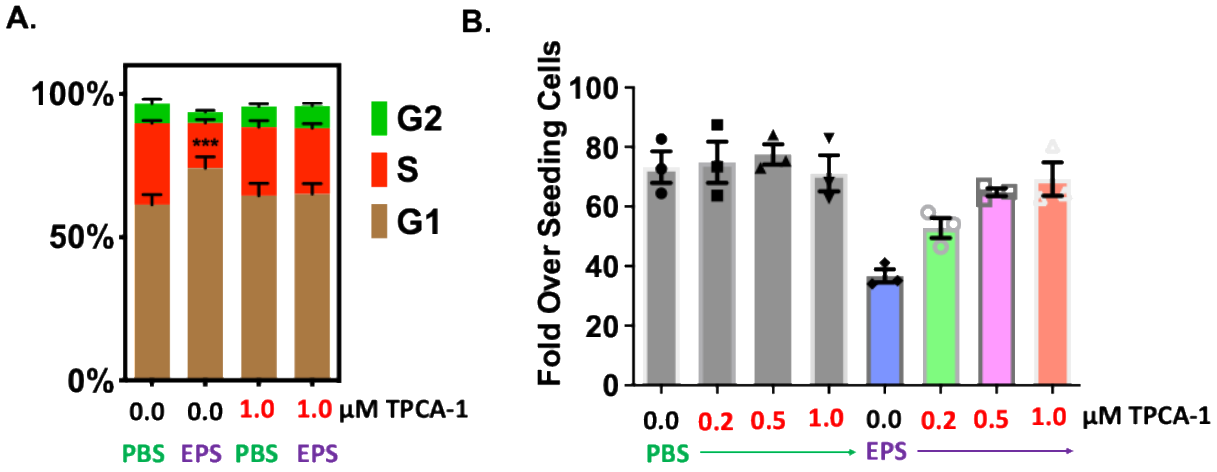


Figure 27. Effects of IKK Inhibitor TPCA-1 on EPS-induced G1 Cell Cycle Arrest and Growth Inhibition of T47D Cells. A. T47D cells were plated in 12-well plates and treated with PBS or 5µg/mL EPS in the presence of DMSO or 1µM TPCA1 for 24h. Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Experiments were performed three independent times. Data are represented as mean ± SEM of 3 independent experiments performed in triplicate, with t-test comparing %S of PBS vs EPS *** $P \leq 0.001$. **B.** Cells were plated in a 12-well plate overnight. Growth assay was performed in the presence of PBS or 5µg/mL EPS, and DMSO vs increasing doses of TPCA-1 for 6 day. Live cells were counted by trypan blue exclusion on a hemocytometer. Proliferation was calculated as Fold over seeding cells = (#Live Cells on Day 6)/(# Live Cells Plated on Day 0). A dose response to compare the toxicity and efficacy of TPCA-1 in rescuing EPS-mediated growth inhibition is shown. Data are represented as mean ± SEM of 3 independent experiments each performed in triplicate. Ordinary one-way ANOVA was performed, with P=0.0008 for EPS group, and not significant for PBS group.

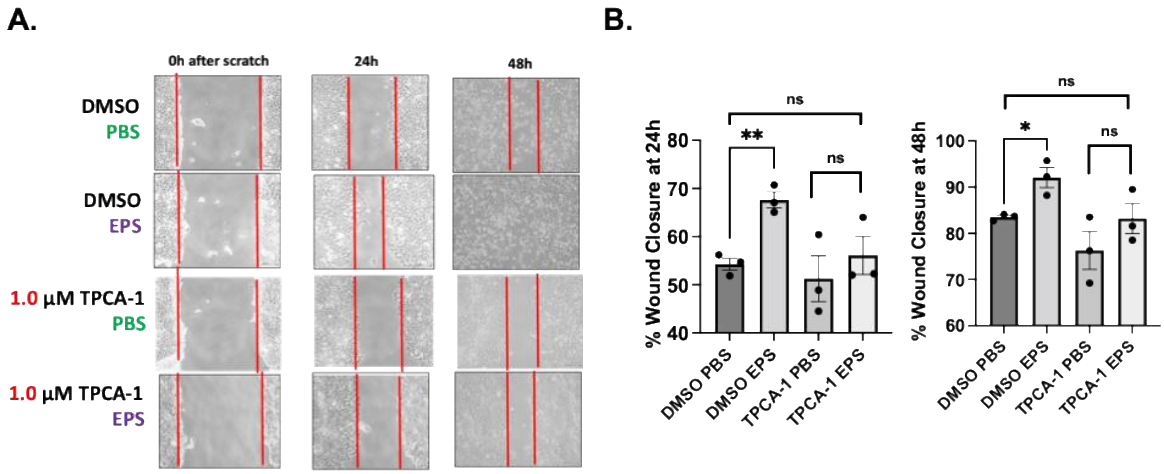


Figure 28. The Effect of the IKK Inhibitor TPCA-1 on EPS-induced Migration of T47D Cells. Cells were pretreated with 1µM TPCA-1 for 30mins, following by 5µg/mL EPS or PBS for 48h until a confluent monolayer of cells was achieved. Then cells were starved in 3% serum overnight. Cells were scratched using a pipette tip and open gaps were imaged at 10X magnification. EPS/TPCA1 treatment was maintained in 3% serum, and the same gaps were imaged again at 24h and 48h. Experiments were performed 3 times. A. Representative images are shown. B. %Wound Closure was quantified using ImageJ. Data are represented as mean ± SEM of 3 independent experiments each performed in triplicate, with t-test * $P \leq 0.05$, ** $P \leq 0.01$, ns = not significant.

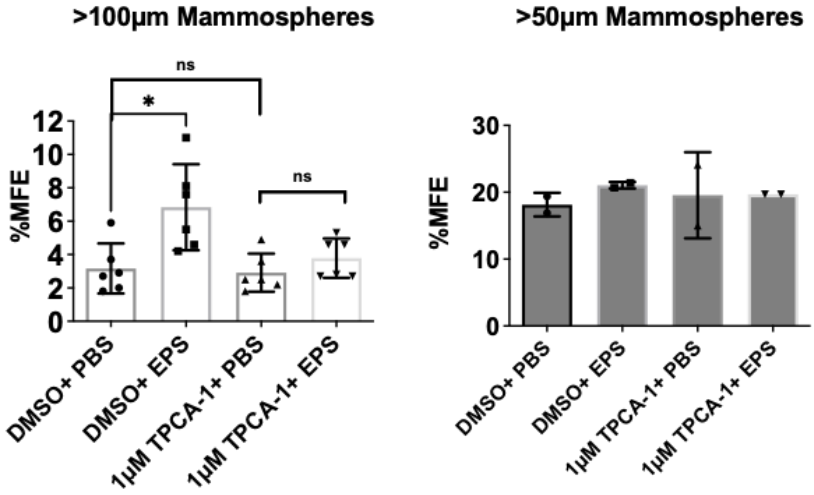


Figure 29. Effect of IKK Inhibitor TPCA-1 and EPS on Mammosphere Formation in T47D Cells. T47D cells were pretreated with 1µM TPCA-1 for 30mins following PBS or 5µg/mL EPS treatment for 4 days. Then, 25,000 single, live cells were plated into mammosphere forming media for 7 days. Mammospheres larger than 100µm were counted and percent mammosphere forming efficiency (%MFE) calculated. Data are represented as mean ± SD of 6 independent experiments, with statistical significance of $P < 0.05$ as calculated using a Student's t-test (Left). On the right, larger than 50µm mammospheres were counted for 2/6 experiments. Data are represented as mean ± SD of 2 independent experiments.

Determining the Target of TPCA-1 in T47D cells: IKKβ versus JAK1

Since TPCA-1 nearly rescued the effects of EPS on T47D cells, we reasoned that the target inhibited by TPCA-1 must be an important player mediating the effects of EPS on breast cancer cells. Thus, identifying the direct target of TPCA-1 would serve to identify the mechanism by which EPS exerted it effects. Since TPCA-1 is known to be highly specific for IKKs, with higher selectively for IKKβ over IKKα, we hypothesized that IKKβ maybe the direct target of TPCA-1 in EPS-treated cells. In addition, TPCA-1 also potently inhibited EPS-induced STAT1 phosphorylation, suggesting that it could inhibit a kinase responsible for phosphorylating STAT1. We hypothesized that JAK1, an important player in the STAT1 pathway, is another potential target of TPCA-1 in T47D cells, as TPCA-1 has been shown to inhibit JAK1 (Cataldi et al., 2015). To test this hypothesis, we performed a knockdown of IKKβ or JAK1 in T47D cells using siRNA and assessed their proliferation and cell cycle progression in response to EPS. If TPCA-1 inhibited either IKKβ or JAK1 to rescue EPS-mediated inhibition of T47D cells, then we expected to see IKKβ or JAK1 knockdown to phenocopy effects of TPCA-1 on proliferation. We found that IKKβ knockdown rescued EPS-mediated cell cycle arrest in the G1 phase and inhibition of proliferation of T47D cells (Figure 30). Treatment with TPCA-1 in scrambled control cells rescued the inhibition of proliferation by EPS and served as a positive control (Figure 30B). In contrast, JAK1 knockdown did not affect either cell cycle arrest or proliferation, as these cells continued to be inhibited by EPS (Figure 30). These data suggest that IKKβ is required for EPS-mediated cell cycle arrest and inhibition of proliferation of T47D cells. Additional studies are needed to further delineate the role of IKKβ in EPS-induced phenotypes in breast cancer cells.

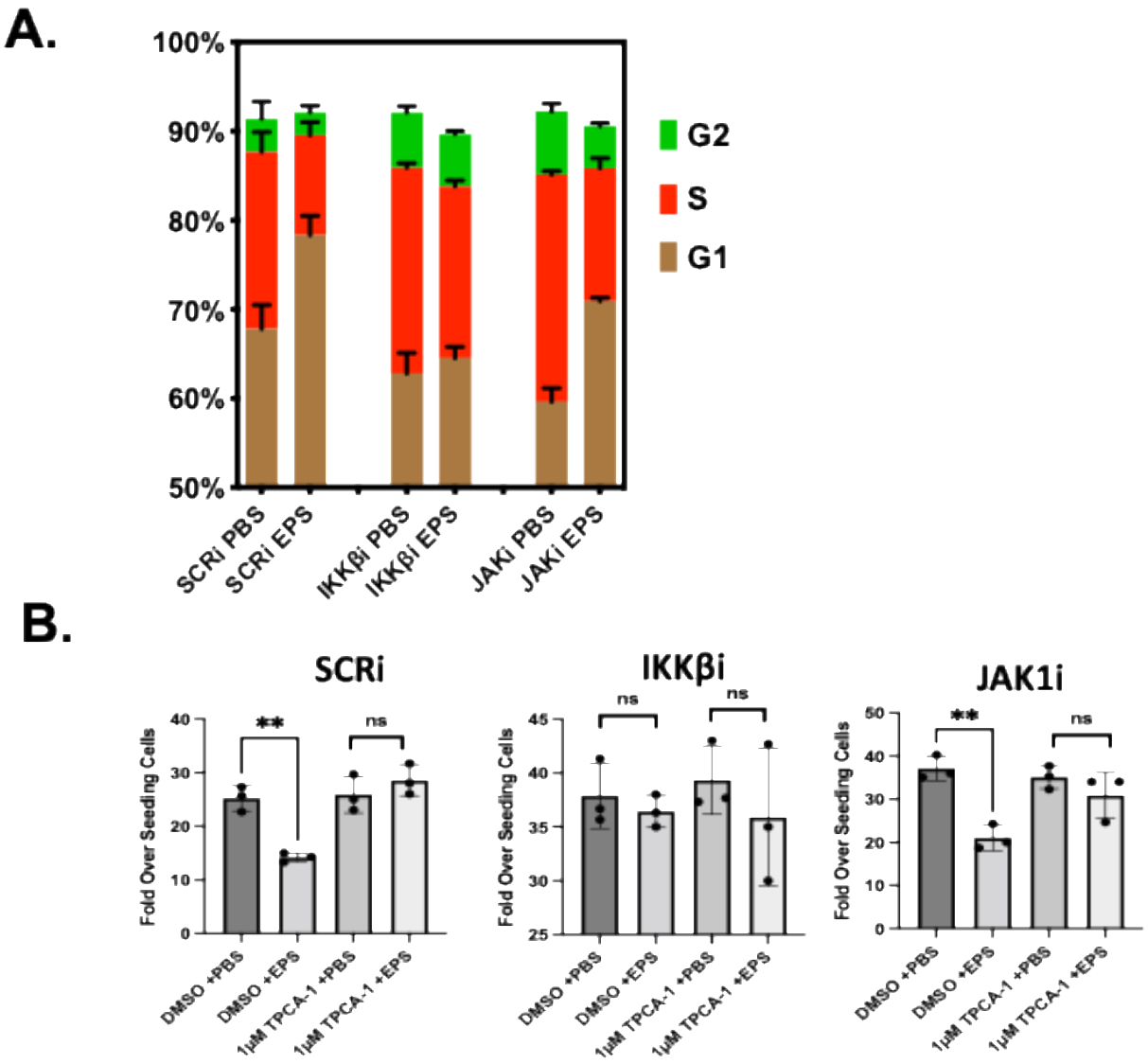


Figure 30. Requirement of IKKβ and JAK1 for EPS-mediated Cell Cycle and Growth Arrest of T47D Cells. Cells were transfected with a smartpool of 4 siRNAs targeting IKKβ or JAK1 or both for 48h (SCRi = scrambled siRNA vs JAKi = siRNA targeting JAK1 vs IKKβ = siRNA targeting IKKβ). **A.** Transfected cells were plated in 12-well plates and treated with 5μg/mL PBS or EPS for 24h. Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Data are represented as mean ± SEM of 2 independent experiments performed in duplicate. **B.** Growth assay was performed on transfected cells in the presence of 5μg/mL EPS and TPCA1 for 6 day. Live cells were counted by trypan blue exclusion on a hemocytometer. Proliferation was calculated as Fold over seeding cells = (# Live Cells on Day 6)/(# Live Cells Plated on Day 0). Data are represented as mean ± SD for one experiment performed in triplicate. t-test was performed for significance. ** $p \leq 0.01$.

Determining the Role of Canonical NF-κB/P65 in EPS-Mediated Cell Cycle Arrest

NF-κB was one of the top transcription factors induced by EPS in sensitive cell lines (Figure 18), and EPS activated the canonical NF-κB within minutes (Figure 20). TPCA-1, a known inhibitor of NF-κB, is able to rescue all the phenotypes induced by EPS in T47D cells. IKKβ, the leading target of TPCA-1, seems to be required for EPS-mediated growth inhibition. Taken together, we then asked if EPS required the p65 subunit of NF-κB, which is downstream of IKKβ to mediate cell cycle arrest. We performed a pilot study in which the p65 was knocked down using siRNA in T47D cells and then assessed their cell cycle response to EPS. If EPS required the canonical NF-κB/p65 subunit to induce G1/G0 cell cycle, then we would expect knockdown of p65 to prevent EPS-mediated cell cycle arrest. However, EPS induced G1/G0 cell cycle arrest regardless of p65 knockdown (Figure 31), suggesting that p65 is not required for mediating cell cycle arrest by EPS in T47D cells.

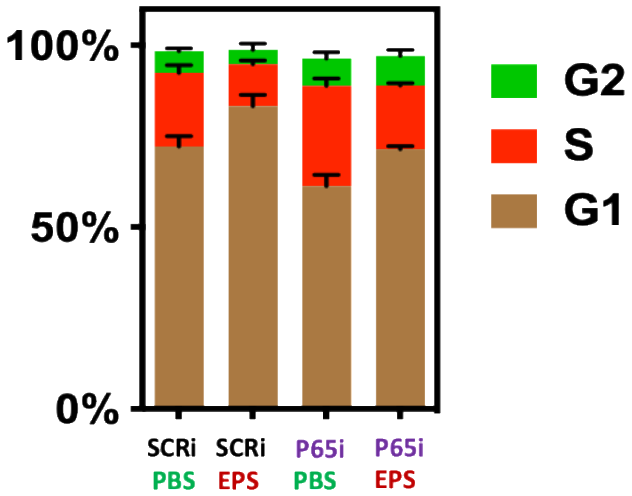


Figure 31. Effect of Knocking Down Canonical NF-κB P65 Subunit on Cell Cycle in the Presence of EPS in T47D Cells. T47D cells were transfected with a smartpool of 4 siRNAs targeting P65 for 48h (SCRi = scrambled siRNA vs P65i = siRNA targeting P65). Transfected cells were plated in 12-well plates and pretreated with 5µg/mL EPS or PBS for 24h. Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with Flow Jo. Data are represented as mean ± SEM of 3 independent experiments performed in triplicate

Determining the Role of STAT1 Signaling in EPS-Mediated Cell Cycle Arrest

The Interferon/STAT1 pathway is among the top pathways induced by EPS in T47D cells (Figure 17). We confirmed the activation of the STAT1 pathway via western blot, showing EPS increased phosphorylation of STAT1 at tyrosine 701 in T47D cells (Figure 19). TPCA-1, a known inhibitor of IKKβ and JAK1, inhibited the activation of both NF-κB and STAT1 and rescued all 4 cancer-associated phenotypes induced by EPS (Figure 26-29). Although IKKβ is required by EPS to induce cell cycle arrest (Figure 30), the canonical NF-κB/p65 subunit downstream of IKKβ was not required (Figure 31). Thus, the remaining pathway that was highly activated by EPS - the Interferon/JAK-STAT remains to be explored. We hypothesized that the STAT1 pathway activated by EPS to induce cancer-associated phenotypes in T47D cells.

We first investigated the canonical Interferon/JAK-STAT pathway in which interferon binds to the interferon receptor resulting in activation of JAK1 and phosphorylation of STAT1 (Khodarev et al., 2012). Although TPCA-1 is known to inhibit JAK1, we showed that JAK1was not necessary for EPS to induce cell cycle arrest in T47D cells. (Figure 30). We then asked whether interferon-gamma (IFNɣ), a potent activator of STAT1, could also induce cell cycle arrest in T47D cells in a similar fashion to EPS. The hypothesis was that EPS induced the expression of an interferon, which then binds to a receptor and activates STAT1. For the first part, we did not detect any IFNɣ in the medium of EPS-treated cells using CBA (Figure 22 and Figure 23). We also found no upregulation of IFNs mRNA in our RNA-SEQ data. To answer the second part of the hypothesis, we treated T47D cells with either EPS or 10ng/mL IFNɣ for 24 hours followed by cell cycle analysis. Results showed that although

IFNɣ potently and quickly activates STAT1, it does not induce G1/G0 cell cycle arrest similar to EPS (Figure 32). The possible reason for this is that EPS activated STAT1 within 2-3 hours while IFNɣ activated STAT1 within minutes. Thus, EPS may require upregulation of other genes or pathways that converge on STAT1 signaling independent of IFNɣ. We have not yet tested type I IFNs (IFN-α and/or -β) as these lead to STAT1/STAT2 heterodimers. However, we have not observed activation of STAT2 by EPS. Taken together, these findings suggest that EPS may activate STAT1, but may be through a pathway independent of canonical Interferon/JAK-STAT pathway.

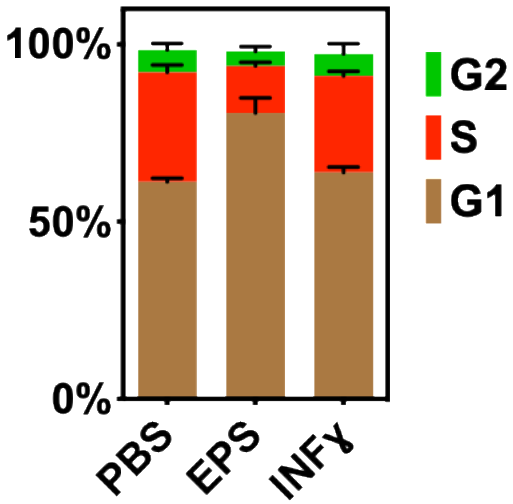


Figure 32. Effects of IFNɣ and EPS on T47D Cell Cycle Progression. T47D cells were treated with 5µg/mL EPS and/ or 10ng/mL IFNɣ for 24h. Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Data are represented as mean ± SEM of 2 independent experiments performed in triplicate.

To address whether the phosphorylation and activation of STAT1 is required by EPS to induce changes in T47D cell phenotypes, we first attempted to knockout STAT1 using CRISPR/Cas9. However, we were unable to obtain a clone that had a complete STAT1 knockout (Figure 30A). It could be that STAT1 is essential in these cells, and STAT1-

deficient cells died. The few surviving clones that showed decreased total STAT1 were still growth inhibited by EPS (Figure 33B), as EPS was shown to induce phosphorylation of the remaining STAT1 in these clones (Figure 33A). Similarly, an RNAi knockdown approach using a smartpool of 4 siRNAs targeting STAT1 decreased total STAT1, but was unable to prevent STAT1 phosphorylation induced by EPS (Figure 34A). Furthermore, mRNAs of STAT1-driven genes (STAT1, P21, IRF1) continued to be increased in response to EPS in T47D cells knocked down for STAT1, indicating that STAT1 was functional (Figure 34B). In this single experiment, we used IFNɣ, a classical activator of STAT1 signaling, as a positive control. Overall, these data indicated that knockdown of total STAT1 was inefficient as the remaining STAT1 could still functions, and could not be utilized to determine if STAT1 phosphorylation was required for EPS-mediated inhibition of T47D proliferation.

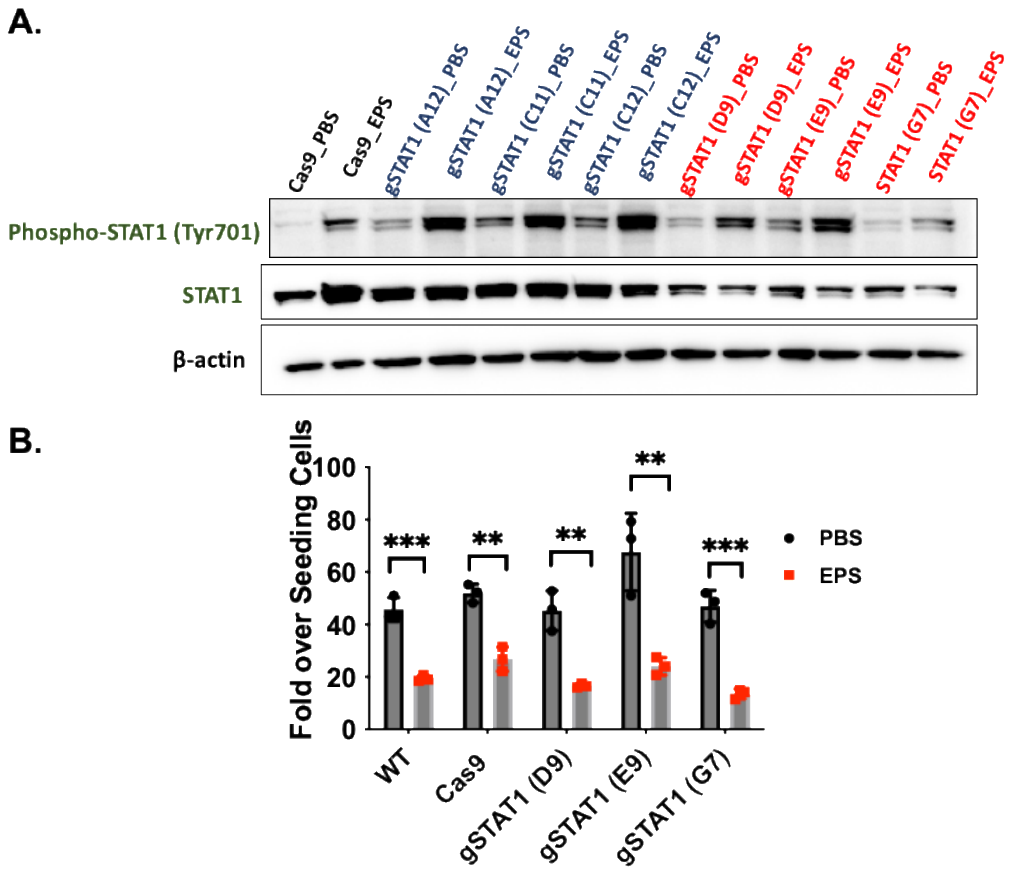


Figure 33. Effect of EPS on CRISPR/Cas9 Clones of T47D Cells with Reduced STAT1. A. sgRNA was designed to knockout STAT1 in T47D cells, cloned into a Cas9-expressing vector and transfected into T47D cells. Pooled cells were cloned and various gSTAT1 clones were tested via western blot for expression of STAT1 and its phosphorylation in response to 3h EPS treatment. ß-actin was used as a loading control while Cas9-only cells were used as control for STAT1 expression. **B.** Growth assay was performed for 3 gSTAT1 clones, wild-type and cas9-control in the presence of PBS, 5µg/mL EPS for 6 days. Live cells were counted by trypan blue exclusion on a hemocytometer. Proliferation was calculated as Fold over seeding cells = (# Live Cells on Day 6)/(# Live Cells Plated on Day 0). Data are represented as mean ± SD for one experiment performed in triplicate. Student's t-test was performed to calculate statistical significance. **P ≤ 0.01 *** ≤ 0.001.

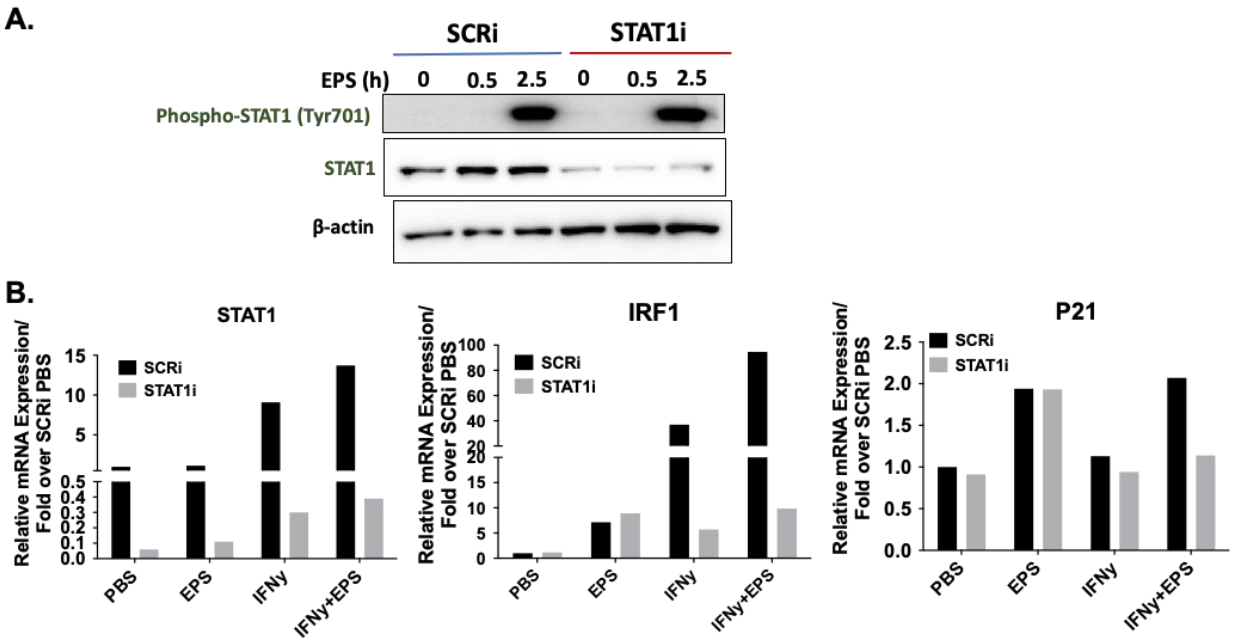


Figure 34. EPS-induced Phosphorylation of STAT1 and STAT1-driven Genes in RNAi Knockdown of STAT1 in T47D Cells. T47D cells were transfected twice with a smartpool of 4 siRNAs targeting STAT1 for 48h (SCRi = scrambled siRNA vs STAT1i = siRNA targeting STAT1). A. Transfected cells were treated with PBS or 5µg/mL EPS for 0, 0.5h, 2.5h and lysates were collected. Western blot was performed to detect protein expression of phosphorylated STAT1 and total STAT1. ß-actin was used as a loading control. Experiments were performed at least 3 independent times, and a representative image for each protein is shown. **B.** Transfected cells were treated with PBS, 5µg/mL, or 10ng/mL IFNɣ (positive control for STAT1-induced genes) for 24h. RNA was collected and qPCR was performed for known STAT1-regulated genes, including STAT1, IRF1, and P21. Relative expression of mRNA was normalized to HPRT, and relative to the scrambled control (SCRi) PBS using the $2^{-\Delta\Delta Ct}$ calculation. Data are from one qPCR experiment.

Both genetic knockout or knockdown approaches directed at STAT1 were unsuccessful at completely depleting STAT1. However, incomplete knockdown was also not useful because the small amount of STAT1 protein remaining was phosphorylated in response to EPS. Hence, in order to address the role of STAT1 in EPS-induced cancer phenotypes, a pharmacologic approach was taken to inhibit STAT1 indirectly by targeting its kinase. We used Cerdulatinib, a known JAK1 inhibitor with promiscuous activity towards other kinases. One of the other top targets of this inhibitor is IKK, which was required by EPS to induce cell

cycle arrest. This inhibitor at 1μM successfully inhibited STAT1 phosphorylation in the presence of EPS (Figure 35A). Cerdulatinib at this concentration also inhibited STAT3 phosphorylation (Figure 35A), but had no effect on NF-κB activation as measured by levels of phosphorylated p65 (Figure 35B). Using Cerdulatinib at the concentration which inhibited phosphorylation of STAT1 (1μM), we performed cell cycle analysis of T47D cells in the presence of PBS or EPS. If EPS required phosphorylation of STAT1 to induce cell cycle arrest in T47D cells, then we expected that Cerdulatinib would prevent EPS-induced STAT1 phosphorylation and cell cycle arrest. Indeed, Cerdulatinib at 1μM or 3μM rescued the G1/G0 cell cycle arrest induced by EPS (Figure 36). However, Cerdulatinib was found to be toxic as assessed by proliferation assays (Data not shown). Thus, effects of Cerdulatinib on EPS-mediated proliferation were inconclusive.

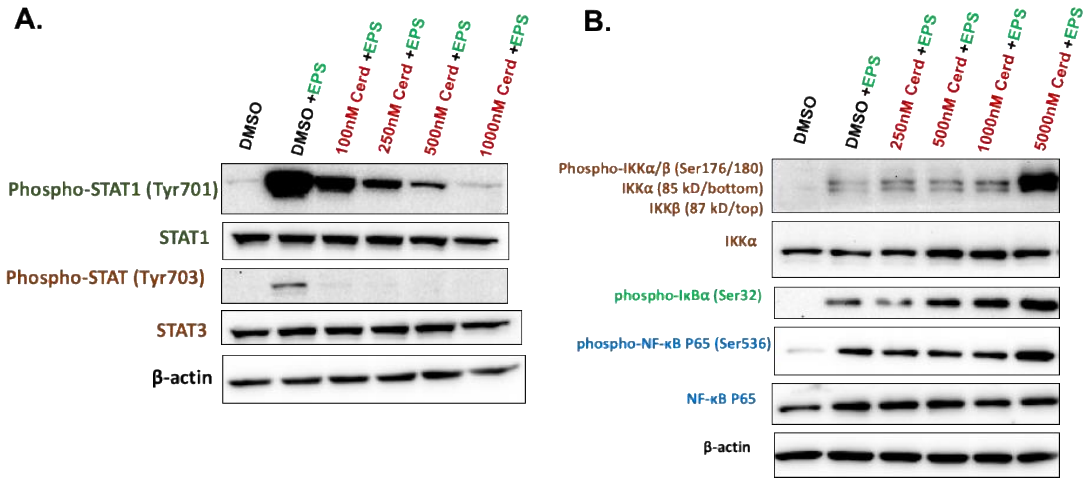


Figure 35. STAT1, STAT3 and NF-κB Phosphorylation Patterns in Response to the JAK1/Pan Tyrosine Kinase Inhibitor (Cerdulatinib) in EPS Treated T47D Cells. T47D cells were pretreated with increasing concentrations of Cerdulatinib (Cerd) for 30min, then treated with 5µg/mL EPS for an additional 2h. Total cell lysates were collected and proteins were detected by western blot using antibodies against indicated proteins. Expression of PY701-STAT1, total STAT1, PY703STAT3, total STAT3, P-IKKα/β, total IKKα, P-IκBα, P-NF-κB, total NF-κB and loading control ß- actin proteins in response to DMSO, EPS, EPS plus increasing dose Cerd. **A.** A dose-response of Cerd in the presence of EPS to demonstrate the lowest concentration that eliminated the P-STAT1 induced by EPS. **B**. A dose-response of Cerd in the presence of EPS to demonstrate the effects on the NF-κB signaling pathway. Experiments were repeated twice. A representative image for each protein is shown.

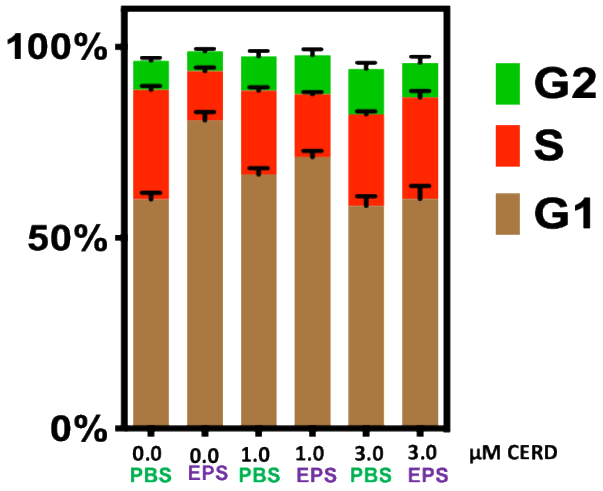


Figure 36. Effect of JAK1/Pan Tyrosine Kinase Inhibitor Cerdulatinib on EPS-induced G1 Cell Cycle Arrest of T47D Cells. T47D cells were plated in 12-well plates and treated with PBS or 5µg/mL EPS in the presence of DMSO vs 1µM or 3µM CERD (Cerdulatinib) for 24h. Cells were fixed and stained with propidium iodide. Cell cycle analysis was performed with FlowJo. Data are represented as mean ± SEM of 3 independent experiments each performed in triplicate.

Conclusions on Mechanism of EPS

EPS induced various phenotypes upon breast cancer T47D cells, from cell cycle arrest to growth inhibition to increased migration, mammosphere formation, and tumor growth. The mechanism by which EPS exerts these effects on breast cancer cells is not well-understood. The data presented here demonstrate that the chemical inhibitor TPCA-1 was able to rescue all the phenotypes induced by EPS in T47D cells *in vitro*, suggesting it must have targeted a central regulator induced by EPS. TPCA-1 was shown to inhibit EPS-induced activation of both STAT1 and NF-κB. TPCA-1 was a potent, selective inhibitor of IKKβ. IKKβ was required for EPS to induce cell cycle arrest, but its downstream canonical NF-κB/P65 subunit was not required by EPS. On the other hand, Interferon/JAK1 was not required by EPS to induce cell cycle arrest, but its downstream regular STAT1 seemed to play an important role. Additional clue come from the pan-tyrosine kinase inhibitor Cerdulatinib which inhibited both IKKβ and JAK1, similar to TPCA-1. Treatment with Cerdulatinib inhibited EPS-induced STAT1 activation with minimal effect on the NF-κB pathway and rescued cell cycle arrest induced by EPS. Taken together, these findings suggest that activation of both IKKβ and STAT1 play critical roles in EPS-induced cancer-associated phenotypes. As IKKβ is not a tyrosine kinase and has not been shown to directly interact with STAT1, additional studies are needed to fully determine the mechanism by which EPS activates these pathways.

CHAPTER FOUR

DISCUSSION

Proposed Model of How EPS Regulates Breast Cancer Cell Phenotypes

Based on data thus far, we proposed a working model for how EPS modulates signaling in T47D breast cancer cells to alter their proliferation, migration, and cancer stemness (Figure 37). Although the EPS dose-response curve is very suggestive of a single receptor (Figure 2), the receptor for EPS has yet to be identified. As EPS appears to be a large molecule (> 300kDa) (Jones et al., 2014), it may not necessarily be internalized to initiate signaling cascade. We observed that the NF-κB pathway was activated within minutes of EPS exposure (Figure 20). IKKβ, in particular, seems to be associated with STAT1 phosphorylation at tyrosine 701, likely through interaction with an unidentified tyrosine kinase. The phosphorylation of STAT1, a marker of pathway activation, is critical in mediating EPS-induced G1 cell cycle arrest, inhibition of proliferation, enhanced migration, and survival of breast cancer stem cells. IKKβ inhibitors (Cerdulatinib and TPCA-1) were shown to abrogate EPS-induced STAT1 phosphorylation and subsequent cancer associated phenotypes (Figure 26-29, 35-36). Although EPS stimulates TNFα secretion, which can induce G1 cell cycle arrest in an autocrine fashion, blockade of TNFα alone was insufficient to block EPS-mediated inhibition of cell cycle progression (Figure 22-25).

Most of the work focused on the T47D cells since these cells were convenient and resulted in similar results as the other cell lines. Although we prioritized working with one cell

line at a time due to limited time and resources, our strategy is that when we understand the mechanism in one cell line, we will quickly apply our findings to other sensitive cell lines to determine whether they share the same mechanism. RNA-SEQ analysis included multiple cell lines and we ensured that we focused on the pathways shared by sensitive and not resistant cell lines. STAT1 and IKK are activated across all four sensitive cell lines. Although these cell lines are very different from each other, we reason that EPS would retain the critical piece of mechanism at least. Having a guide to start somewhere would speed up our process on other cell lines.

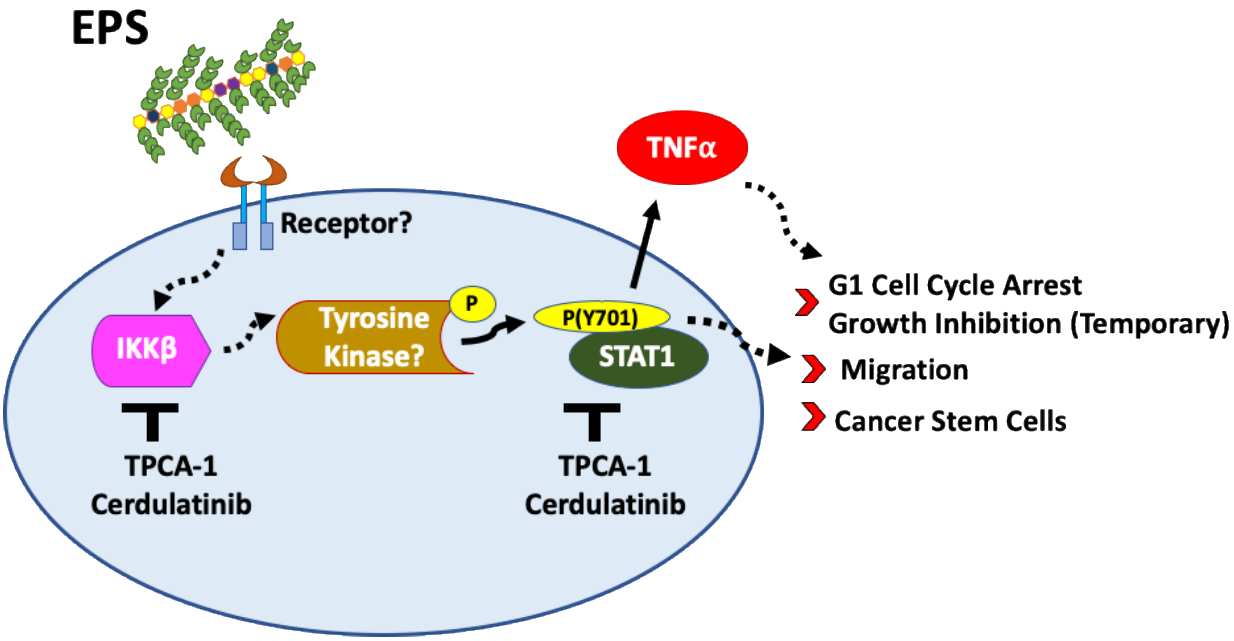


Figure 37. Working Model of the Mechanism by which *B. subtilis* EPS Modulates Cancer Associated Phenotypes of T47D Breast Cancer Cells. Exogenous EPS binds to an unidentified receptor and activates the NF-κB pathway, particularly IKKβ. IKKβ then through an unknown mechanism activates a tyrosine kinase which phosphorylates STAT1. EPS also upregulates TNFα production and secretion, which plays a role in arresting cells in the G1 phase of the cell cycle. STAT1 is also critical in enhancing migration and cancer stem cell survival through a mechanism yet to be determined.

Potential Link between IKKβ, Tyrosine Kinase and STAT1

STAT1 activation requires the phosphorylation of the tyrosine 701 residue and subsequent homo or heterodimerization (Wenta et al., 2008). Although we showed that phosphorylation of STAT1 at tyrosine 701 is important for phenotypes induced by EPS in T47D cells, we have yet to determine which tyrosine kinase phosphorylates and activates STAT1 in the presence of EPS. The canonical JAK/STAT pathway involves interferon binding to the interferon receptor and subsequent activation of JAK resulting in phosphorylation of STAT1 (Nan et al., 2017). However, our data showed that activation by interferon-γ in T47D cells did not induce cell cycle arrest or inhibit proliferation to the same degree as with EPS (Figure 32). Furthermore, knocking down JAK1 via siRNA did not interfere with EPS-mediated inhibition of cell cycle progression (Figure 30), suggesting that EPS may not utilize the canonical Interferon/JAK/STAT1 pathway. We know that a tyrosine kinase is involved, as the pan-tyrosine inhibitor Cerdulatinib and the IKK inhibitor TPCA-1 abrogated STAT1 phosphorylation and EPS-mediated cancer-associated phenotypes (Figure 26-29, 35-36). Both of these inhibitors target IKKβ, and initial studies show that knocking down IKKβ seemed to rescue EPS-mediated growth inhibition (Figure 30). Curiously, TPCA-1 (a potent and selective inhibitor of IKKβ >> IKKα) induced higher levels of phospho-IKKα/β in a dose dependent manner (Figure 26A). While NF-κB signaling appeared dampened in the presence of TPCA-1, the more impressive inhibition was seen in the levels of phospho-STAT1 (Figure 26B). Thus, more experiments are needed to delineate whether IKKβ is the target inhibited by TPCA1 to rescue EPS-mediated phenotypes, and how IKKβ is involved with STAT1 phosphorylation by EPS.

IKKβ is a serine/threonine kinase known to phosphorylate IκBα, leading to IκBα degradation and nucleus translocation of NF-κB (Mitchell et al., 2016). Being a serine/threonine kinase, it is unlikely that IKKβ would be able to phosphorylate directly the 701-tyrosine residue on STAT1. Thus, an unidentified tyrosine kinase may be involved. IKKα, which is the sister kinase to IKKβ within the IKK complex (Liu et al., 2012), may also need to be investigated to see whether it plays a role in EPS signaling.

We elected to knockdown IKKβ first because TPCA-1 has a 22-fold selectivity for IKKβ over IKKα (Podolin et al., 2005). Interestingly, one study showed that silencing of IKKα significantly decreased STAT1 tyrosine phosphorylation in response to dsRNA in HeLa cells (Xing et al., 2016). Xing et al suggested that IKKα can mediate both type I interferon-dependent and interferon-independent STAT1 phosphorylation during an antiviral response. However, no physical interaction between IKKα and STAT1 was detected (Xing et al., 2016). Otherwise, it is completely unknown how IKKβ or IKKs would modulate tyrosine phosphorylation of STAT1 in an interferon-independent manner. Potentially some other tyrosine kinase besides the classical JAK1/JAK2/TYK may be responsible for phosphorylating STAT1 in the presence of EPS. For example, SYK has been shown to mediate the tyrosine phosphorylation of STAT1 via the RIG-I/MAVS/SYK pathway during innate antiviral immunity (Liu et al., 2021).

EPS and Commensal Bacteria in the Context of Breast Cancer and Health

As the microbiome has been recognized as being part of the tumor microenvironment, so has dysbiosis induced by various factors been associated with breast cancer development (Rao Malla et al., 2022). As the development is very recent, the field investigating the role of bacteria in breast cancer is still in its infancy. Microbiome studies tend to report large-scale change in

bacterial composition, which makes it difficult to pinpoint the specific causal microbes. So far there have been few reports regarding specific commensal bacteria and breast cancer. Thus, our study of *B. subtilis* EPS is among the first to examine how probiotic bacteria and the molecules they produce may impact breast cancer. Although widely included in many probiotic preparations, there was virtually no information published regarding *B. subtilis* and breast cancer.

Putting together what we learned so far, we proposed a preliminary model in which *Bacillus subtilis* and the EPS it produces may impact breast cancer and health (Figure 38). Initially, people are exposed to *B. subtilis* through contact with soil or hay in the environment or through consuming of traditionally fermented food or probiotics. As *B. subtilis* colonizes the gut and produces EPS, it may exert local and systemic effects on the immune system, creating a healthy anti-inflammatory state as a commensal bacterium. EPS may also travel to breast tissue, interacting directly with breast cancer cells to modulate their growth and behaviors. As EPS was found to promote breast tumor growth in mice, it may also promote breast cancer initiation and/or progression in humans. However, data are lacking to make any conclusions. More experiments are needed to determine how physiologically relevant is the role of EPS in the human population in general and in breast cancer patients in particular. As the working mechanism of EPS is further elucidated, we could identify which breast cancer subtype or patient population would be more at risk due to *B. subtilis*/EPS exposure.

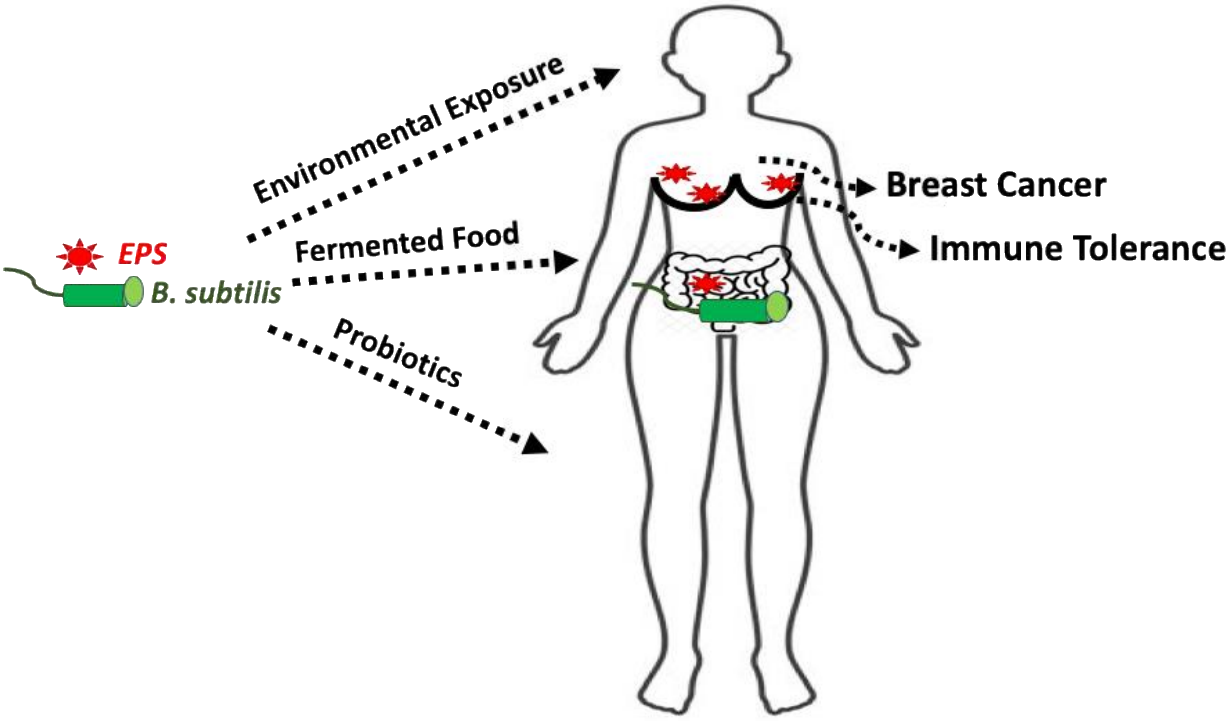


Figure 38. Proposed Model of the Effects of *B. subtilis* and its EPS on Human Health. Humans can be exposed to *Bacillus subtilis* via their environment (soil, hay), fermented food or commercial probiotic preparations. *B. subtilis* colonizes the gut and produces EPS. EPS has systemic effects and may localize to the breast tissue. Within the breast microenvironment, EPS can induce an anti-inflammatory state on immune cells and/or act directly on breast cancer cells to modulate tumor growth.

Differences *in vitro* versus *in vivo* and Implications for Drug Development

One surprising finding from this study is that growth inhibition *in vitro* may not translate to a reduction in tumor growth *in vivo*. We utilized commonly used assays to measure tumor properties including a viability assay (XTT), cell cycle progression (propidium iodide – flow cytometry), rates of live cell proliferation, and cell death analysis (Annexin V-flow cytometry). EPS appeared as a potent anti-proliferative agent across all these *in vitro* assays. Many studies assess the cytotoxicity of the agents *in vitro* and stop there. Another caveat is that studies may only focus one particular phenotype such as proliferation or migration, while a single drug may have different effects depending on the measured phenotype. Hence, reports of *in vitro* data

could be incomplete or misleading. For a novel drug/agent, full characterization across different cancer phenotypes is necessary to understand how it affects cancer behavior to guide proper usage.

Furthermore, *in vitro* findings may change when tested *in vivo* using an intact mouse model. There are certainly more factors at play *in vivo* that could alter the tumor's response to drug, from drug bioavailability to other cell types being affected. This further highlights the importance of preclinical testing *in vivo*. Going from mice to humans is another large step that introduces more variables, which partly explains why so many drugs failed in clinical trials. Lastly, many drugs are strictly studied in the context of the disease that they are intended to treat, leaving them vulnerable to unexpected side effects as other systems within the body can also be affected. In the case of EPS, it is being explored as a probiotic treatment for inflammatory diseases. However, our findings revealed that EPS has multiple modes of action leading to tumor growth rather than tumor regression. Thus, caution may be needed for use of EPS in humans at high risk for breast cancer, being treated for breast cancer, or having completed treatment for breast cancer.

Challenges and Limitations of EPS

Based on RNA-SEQ data and our experimental results, *B. subtilis* EPS activates multiple pathways in breast cancer cell lines that are responsive to it, including NF-κB, STAT1, STAT3, P38/MAPK, and TNFα. However, many genes and pathways upregulated by EPS treatment were not required for EPS-mediated cancer-associated phenotypes, except for IKK and STAT1 signaling. It is challenging to interrogate each pathway to delineate the exact function it plays in

different phenotypes. Moreover, these pathways may crosstalk with each other redundancy, making targeting a single protein/pathway insufficient to rescue EPS-mediated phenotypes.

Another challenge with EPS is that the structure of EPS is not completely elucidated, and the composition of EPS may vary from batch to batch. Unlike a synthesized protein or purified chemical, EPS is purified from *B. subtilis* secretion/biofilm. At the end of the isolation process, we likely have a mixture of carbohydrates depleted of RNA, DNA and protein. However, we do not know exactly what carbohydrates we have from batch to batch. It is well known that bacteria can make different biofilm compositions of EPS depending on the growth condition (Vu et al., 2009). Although our EPS purification has been standardized and we strictly follow the same steps each time, we still experience variation in the efficacy of EPS from batch to batch. Another variable is the concentration of EPS used. We performed a carbohydrate assay using sulfuric acid to measure the concentration of EPS. However, this is the total amount of carbohydrates within the mixtures. The actual concentration of active carbohydrates may vary in the mixture and may not be accurate.

Lastly, we have been focusing on direct effects of EPS on some cancer cells. As breast cancer is a heterogeneous disease, it is unclear the relevance of these findings for breast cancer patients with different subtypes and pathogenesis. More experiments are needed to determine what cell types would respond to EPS. Additionally, our experiment used concentrated EPS that is likely not found under physiological conditions, and it is unknown whether EPS can actually localize to cancer cells within the breast tumor in humans. Future experiments using *B. subtilis* in immunocompetent mouse models listed below will help to address these points.

Future Directions

Determine the Molecular Mechanism of EPS Action in Breast Cancer

There is still much to learn about how EPS mediates its action on breast cancer cells at the molecular level. We have yet to identify the kinase(s) that phosphorylate STAT1 in response to EPS, and how IKKβ is involved with STAT1 phosphorylation. First, we need to examine whether knocking down IKKβ would also abrogate EPS-induced STAT1 phosphorylation, similar to TPCA-1. We can also investigate whether there is a direct interaction between IKKβ and STAT1 using immunoprecipitation, immunofluorescence microscopy, or mass spectrometry. Another potential experiment would be to pull down phospho-STAT1 via immunoprecipitation and identify interacting protein partners using liquid chromatography-mass spectrometry (LC-MS) to identify the tyrosine kinase(s) involved. Additionally, IKKα may also need to be investigated to see whether it plays a role in EPS signaling.

Another point of investigation would be to identify the receptor for EPS in these breast cancer cells. EPS activates multiple signaling pathways in EPS sensitive cell lines while having little effect on resistant cells (Figure 21). This suggests that the sensitive cells may possess a receptor(s) for EPS that resistant cells lack. Moreover, proliferation of T47D cells by a dose response of EPS indicates that there may be a single receptor involved based on the pattern of the proliferation curve (Figure 2). On immune cells, EPS requires TLR4 signaling (Jones et al., 2014; Kalinina et al., 2021; Paik et al., 2020). However, TLR4 is not required for EPS to inhibit the proliferation of T47D cancer cells (Figure 11), suggesting that some other receptor/coreceptor may be necessary. Thus, it is critical to identify the receptor for EPS. Using

these multiple breast cancer cell lines, it is possible to use a labelled EPS to pull down all interacting proteins. Subsequent LC-MS may be used to identify the receptor.

Lastly, we need to confirm whether the same mechanism used by EPS is conserved across different breast cancer cell lines. We showed that EPS stimulates phosphorylation of STAT1 in at least four sensitive breast cancer cells (Figure 19 and 21). Future experiments need to address whether TPCA-1 or IKKβ knockdown also rescue EPS-mediated inhibition of the other breast cancer cells. Understanding how EPS initiates a signaling cascade to promote its phenotype would give us insights to determine what cell type would be affected by EPS and predict the extent to which EPS would impact health of its human host.

Determine How EPS Affects Breast Tumor Growth in the Presence of an Immune System

Our work thus far has focused on direct effects of EPS on breast cancer cells. In cultures, short term treatment with EPS temporarily inhibits proliferation of certain breast cancer cells (Figure 3). Long-term exposure to EPS *in vitro* no longer inhibits cell growth (Figure 14), and EPS *in vivo* promotes breast tumor growth in immunocompromised mouse models (Figure 15 and 16). Additionally, it is well-established that EPS is able to induce an anti-inflammatory state to protect mice from various T-cell mediated diseases (Jones et al., 2014; Kalinina et al., 2021; Paik et al., 2019, 2020; Paynich et al., 2017; Swartzendruber et al., 2019). As the immune system is known to play important roles in immune surveillance and immunoediting of tumors, it is important to determine how EPS affects growth of breast tumors in the presence of a functional immune system. There are two approaches to investigate this: syngeneic murine models and humanized mouse models. Using a syngeneic murine model would require a screen for a murine breast cancer cells that respond to EPS treatment. We could implant these EPS-treated murine

cancer cells into immunocompetent mice and monitor tumor growth. One caveat here is that a mouse tumor and mouse immune response may be different compared to that of humans. This is precisely the reason why xenograft models using human breast cancer cells/cell lines derived from patient' tumors are favored. Another potential difficulty would be finding a murine cell line that responds to EPS, as we have yet to determine which characteristics of a cell are required to respond to EPS. One common murine breast tumor cell line, 4T1, was tested and was unaffected by EPS. Another approach would be to use human xenografts in mice with a humanized immune system. Humanized mice permit transplantation of human xenografts and allow for tumor growth. Mice would be irradiated to eliminate the host's immune system, and then reconstituted with a human immune system. The advantage here is that we can use the same human breast cancer cells that we have characterized and study their response to EPS. Disadvantages include technical challenges with more steps involved in creating the Model, and potential issues with engraftment of the human immune system. We would expect that as EPS generated a suppressive immune environment, growth of breast tumors would be enhanced rather than suppressed in immunocompromised mice.

Additionally, *in vitro* experiments could be carried out to study how EPS affects the ability of cytotoxic T-cells and NK cells to kill breast cancer cells. Multiple killing assays which measure killed target ratio, chromium release or cytokine release can be utilized to measure the killing capacities of these effector cells. It would be interesting to see whether direct treatment of EPS *in vitro* alters effector cell killing capacities. Based on previous studies by the Knight lab, EPS may require other cell types *in vivo* to modulate T cell activities (Jones et al., 2014; Kalinina et al., 2021; Paik et al., 2019, 2020; Swartzendruber et al., 2019). Thus, we can also isolate

CD8+ T cells and NK cells from EPS-treated mice to use in the killing assay. As EPS is known to inhibit T cell activation, we would expect that EPS would reduce effector cell killing capacities. However, our RNA-SEQ data indicated that EPS treatment upregulated MHC I on breast cancer cells (Data not shown), which could make them more visible to NK cells.

Another venue to investigate would be to see how EPS may impact immunotherapy. It was shown that immunotherapy such as checkpoint inhibitors required the microbiota (*B. fragilis* in particular) to work effectively. As EPS tends to induce immune tolerance, we would expect that checkpoint inhibitor immunotherapy may not work as well on breast cancer in the presence of EPS. However, the outcome of immunotherapy may be influenced by multiple factors.

Determine How Colonization with *B. subtilis* would Affect Breast Tumor Growth

Investigating *B subtilis* EPS simplifies studies on determining the mechanism of action in breast cancer cells. However, using the *B. subtilis* bacterium to investigate the role of this probiotic on breast tumor growth would be more physiologically relevant. Most people are colonized by *B. subtilis*, and are not likely exposed to EPS at such high concentrations as used in this study. Thus, we would want to colonize the gut of immunocompetent mice with *B. subtilis* spores and determine the impact on growth of breast tumor xenografts. Here, we should start with a breast cancer cell line that we know responds to EPS *in vitro*. Furthermore, we may want to use spores from *a B. subtilis* strain that is either wild-type, EPS-overproducing, or EPS lacking to determine the impact of EPS from the bacterium as a whole. Mice can also be given broad-spectrum antibiotics before receiving spores to investigate whether dysbiosis of the gut microbiome may be necessary for *B. subtilis* to exert a phenotype. Lastly, it would be intriguing to investigate whether EPS can be found in breast tissue of mice colonized with *B. subtilis*. This

would require a working antibody that can recognize EPS for IHC or IF staining. These findings would link *B. subtilis* and its EPS with breast cancer in a more physiologic setting relevant to breast cancer patients.

Concluding Statement

Research on the microbiome has changed the way in which we think about the human body and how it functions. Now we are thought of as a supra-organism, comprising of our own cells and our unique microbiome. Likewise, the tumor microenvironment is not just our own cells and the extracellular matrix. The microbiome has also been recognized to play important roles in tumor progression and response. It is fascinating that the bacteria we each carry could contribute to our cancer, for better or for worse depending on the context. Unlike our own cells which are orderly and programmed to behave certain ways, the microbiome is more dynamic, chaotic, unpredictable, and malleable by so many different factors. Studies have barely scratched the surface on ways in which bacteria influence our body and health. Our study presents a novel finding in which a probiotic bacterial product, EPS directly alters breast cancer cell signaling and promotes tumor growth in immunocompromised mouse models. It is not so often that we think of a well-established probiotic/commensal bacterium like *Bacillus subtilis* as a tumor promoter. This goes on to highlight how critical it is to study in multiple contexts and cross-discipline. Our hope is that the more we expand our knowledge, the more we can add to our toolbox and devise better strategies to prevent and treat breast cancer.

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VITA

Mai Rachel Nguyen was born in Gia Kiệm, Việt Nam on June 30th, 1993 to Michael Nguyễn and Teresa Lê. In 2007, she moved with her family to St. Louis, MO and started high school there. Mai attended Washington University in St. Louis, where she earned her Bachelor of Arts, *magna cum laude*, in Biology in May 2015. After graduation, she stayed at the university to work as a lab technician for Dr. Katherine Fuh, studying the roles of receptor tyrosine kinases in endometrial cancer. In 2016, Mai matriculated into the MD/PhD program at Loyola University Chicago Stritch School of Medicine. Following the completion of two years of medical school, Mai began her graduate education in the Microbiology and Immunology Program under the joint mentorship of Dr. Clodia Osipo and Dr. Katherine Knight.

Mai's dissertation work focused on the novel roles in which a probiotic bacterial product (exopolysaccharide isolated from *Bacillus subtilis*) modulates breast cancer. This work was supported by the T32 Immunology Training Grant awarded to Dr. Katherine Knight, and the ARCS Foundation's Illinois Chapter Award granted to Mai. Upon completion of her graduate studies, Mai will return to medical school to finish the MD/PhD program training, joining the Stritch Class of 2024.