AMERICAN UNIVERSITY OF BEIRUT

GLYPHOSATE ALTERS MAMMARY LOBULAR AND DUCTAL EPITHELIAL CELL DIFFERENTIATION AND TRIGGERS TUMOR INITIATION EVENTS

by
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ABSTRACT
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Title: Glyphosate Alters Mammary Lobular and Ductal Epithelial Cell Differentiation and Triggers Tumor Initiation Events

Cancer remains a threat for patients around the world, with an estimated 19.3 million people diagnosed in 2020. Specifically, breast cancer has been recently found to be the most prominently diagnosed cancer globally, accounting for 15.5% of deaths related to cancer in females. The cellular change toward oncogenesis is closely linked to environmental factors such as herbicides, tobacco, and alcohol.

Glyphosate is the most widely used herbicide globally, with usage increasing over the years, from 16 million kg spread in the world in 1994 to 79 million kg spread in 2014, including 15% in the United States alone. The International Agency for Research on Cancer (IARC) classified glyphosate as "probably carcinogenic to humans" (Group 2A) in March 2015, based on "limited" evidence of cancer in humans and "sufficient" evidence of cancer in experimental animals.

Although some studies have looked into its effect on the progression of cancer in tumorigenic cells, it is still unknown whether glyphosate may initiate cancer and disrupt differentiation in normal cells. In this study, we opted to investigate the impact of glyphosate in initiating tumor-like phenotypes in non-tumorigenic estrogen-positive lobular mouse (SCp2) and ductular human estrogen-negative (HMT-3522 S1) breast epithelial cells and disrupting differentiation markers. Our results show that long-term glyphosate exposure did not affect growth rate, but enhanced the invasion compared to the untreated control (~8-fold increase upon 10^{-11} M glyphosate; $p < 0.05$), matrix metalloproteinase-9 (MMP-9) release, and triggered a 4-fold miR-183 upregulation (preliminary), the most overexpressed miRNA in early-stage Lebanese breast cancer patients, in SCp2 cells. Long-term as well as short-term glyphosate treatments of human derived S1 cells exhibited an enhanced cell invasion across a reconstituted basement membrane, whereas long-term exposure disrupted lumen formation in their 3D cultures, compared to non-treated cells (~1.3-fold and 1.2-fold increase upon 10^{-5} and 10^{-11} M glyphosate, respectively; $p < 0.05$). Short and long-term treatments with glyphosate disrupted the distribution of cell polarity marker β-catenin from and apical to a basolateral distribution. Moreover, we showed the effect of the herbicide on the differentiation markers of SCp2 and S1 cells by evaluating levels of β-casein expression and assessing the effect of treatment on lumen formation, respectively. β-casein expression significantly decreased upon 10^{-11} M glyphosate treatment ($p < 0.05$). We additionally propose a possible pathway for the effect of glyphosate on the activation of breast cancer-related signaling pathways using Ingenuity Pathway Analysis (IPA) in estrogen-dependent cells.

In conclusion, our findings may provide insight on the function of glyphosate in tumor initiation events, suggesting that such chemicals might "injure" nontumorigenic breast

epithelial cells. Further research may be necessary to fully understand the extent of these effects and to develop alternative methods for weed control that do not rely on glyphosate.

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ABBREVIATIONS

IARC	International Agency for Research on Cancer
MMP	Matrix metalloproteinase
SC	Stem Cell
3D	Three Dimensional
IPA	Ingenuity Pathway Analysis
ECM	Extracellulat Matrix
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
EPA	Environmental Protection Agency
EFSA	The European Food Safety Authority
CSN2	Casein Beta
IGF	Insulin-like growth factor
EGF	Epidermal growth factor
LALBA	Lactalbumin Alpha
SLC2A1	Solute Carrier Family 2 Member 1
HLA	Human Leukocyte Antigen
DRB1	DR Beta 1
CTGF	Connective tissue growth factor
THBS1	Thrombospondin 1
ELF5	E74 Like ETS Transcription Factor 5
STAT5A	Signal Transducer And Activator Of Transcription 5A
STAT5B	Signal Transducer And Activator Of Transcription 5B
TUG1	Taurine Up-Regulated 1
DMEC	Ductal mammary epithelial cells
IDC	invasive ductal carcinoma
MAPK	Mitogen-activated protein kinases
MEC	Mammary epithelial cells
LMEC	Lobular mammary epithelial cells
BM	Basement Membrane
TJ	Tight Junctions
AJ	Adherens Junctions
GJ	Gap Junctions
TNT	Tunneling Nanotubes
ZO	Zonula Occludens
JAM	Junctional adhesion molecule
CRB-3	Crumbs Cell Polarity Complex Component 3
MAGI	Membrane-associated guanylate kinase, WW and PDZ domain-containing protein
HTLV	human T-lymphotropic virus type 1
GJIC	Gap junctional intercellular communication

VEGF	Vascular endothelial growth factor
TSP-1	Thrombospondin 1
EMT	Epithelial-to-mesenchymal transition
APC	Adenomatous polyposis coli
PTEN	Phosphatase and Tensin Homolog
CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitors
PI3K	Phosphoinositide 3-kinases
mTOR	Mammalian target of rapamycin
PKC	Protein Kinase C
LGN	Lateral geniculate nucleus
GTP	Guanosine-5'-triphosphate
TP53	Tumor protein P53
CDKN2A,	Cyclin-dependent kinase inhibitor 2A
RB1	Retinoblastoma gene
WHO	World Health Organization
DCIS	Ductal carcinoma in situ
IDC	Invasive ductal carcinoma
HR+	Hormone receptor-positive
HER2	Human epidermal growth factor receptor 2
ER	Estrogen Receptor
PR	Progesterone
FOXA1	Forkhead box A1 Protein
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
AJCC	The American Joint Committee on Cancer
CNV	Copy number variants
DMR	Differentially methylated regions
RARβ2	Retinoic acid receptor beta2
MLH1	DNA mismatch repair protein 1
EZH2	Enhancer of zeste homolog 2
HDAC2	Histone deacetylase 2
MALAT1	Metastasis Associated Lung Adenocarcinoma Transcript 1
BC	Breast Cancer
EGR1	Early growth response factor 1
ITGA9	Integrin Subunit Alpha 9
TIMP2	Tissue inhibitor of metalloproteinases 2
FHL1	Four and a Half LIM Protein 1
UTR	Untranslated region
BET	Bromodomain and Extra-Terminal motif
GAS5	Growth arrestspecific 5

DDT	Dichlorodiphenyltrichloroethane
BPA	Bisphenol A
EPSP	5-enolpyruvylshikimate-3-phosphate
NHL	Non-Hodgkin lymphoma
GBH	Glyphosate-based Herbicides
TET3	Tet Methylcytosine Dioxygenase 3
FBS	Fetal Bovine Serum
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
TBST	tris-buffered saline and Polysorbate 20
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
ANOVA	Analysis of Variance
GM	Growth media
NDM	Non-differentiation media
ESR2	Estrogen Receptor 2
SOD1	Superoxide dismutase 1
HOXB9	Homeobox B9
DNMT1	DNA-methyltransferase 1
RBL2	Retinoblastoma-like protein 2
GPR30	G protein-coupled receptor 30
PKC	Protein kinase C
CLDN1	Claudin 1
MAGUK	Membrane-associated guanylate kinase
DSC3	Desmocollin 3
JNCI	Journal of the National Cancer Institute
miRNA	microRNA

CHAPTER I

INTRODUCTION

Breast cancer is the most prevalent and deadly cancer among women worldwide. While much research has been conducted to identify the risk factors associated with breast cancer initiation, the exact causes of this disease remain largely unknown. Epigenetic alterations, which involve modifications to DNA that do not change the underlying genetic code, are increasingly recognized as important contributors to breast cancer development and progression. Studies have demonstrated that aberrant DNA methylation patterns, histone modifications, and microRNAs are frequently observed in breast cancer tissues and cell lines, suggesting that these epigenetic changes play a key role in regulating gene expression and promoting tumor growth (Fraga et al., 2005; Tung and Gilad, 2013). In recent years, there has been growing concern over the potential role of environmental factors that affect the epigenome in the development of breast cancer, such as chronic stress, nutrition, toxins, and exposure to certain chemicals (Jirtle and Skinner, 2007; Fraga et al., 2005; Tung and Gilad, 2013). One such chemical is glyphosate.

Glyphosate is a widely used herbicide that has been the subject of controversy due to its potential health effects. The International Agency for Research on Cancer (IARC) classified glyphosate as "probably carcinogenic to humans" in 2015, which led to subsequent studies regarding its association with several types of cancer-related diseases, including breast cancer. Numerous studies have reported the presence of glyphosate in various body fluids including blood, urine, and breast milk (Zhang et al. 2019;

Bøhn et al., 2014; Krüger et al., 2013). Several studies have since investigated the potential link between glyphosate exposure and breast cancer, but the findings have been inconsistent (Zhang et al., 2021). Glyphosate has been shown to induce epigenetic changes in both *in vitro* and *in vivo* studies, including alterations in DNA methylation patterns and histone modifications (Duforestel et al., 2019; Kwiatkowska et al., 2017). These changes may in turn affect gene expression and cellular processes relevant to breast cancer development, such as hormone receptor signaling and DNA repair. Other studies have demonstrated that glyphosate exposure can alter gene expression and disrupt hormonal balance, potentially leading to the development of estrogen receptor (ER)-positive breast cancer (Thongprakaisang et al., 2013; Kurebayashi et al., 2018). However, Stur et al. (2019) showed deregulation of 11 canonical pathways in both ER-positive and ER-negative breast cancer cell lines. A recent study using human breast cancer cell lines found that glyphosate exposure led to alterations in DNA methylation and gene expression patterns, which were associated with increased cell proliferation and decreased apoptosis (Mesnage et al., 2019). A case-control study in the United States found that women with the highest glyphosate exposure had a higher risk of breast cancer compared to women with the lowest exposure (Franke et al., 2021). Importantly, Gasnier (2009) et al. showed that glyphosate exposure *in vitro* led to changes in cell proliferation, as well as disruptions in the expression of genes involved in mammary gland development and differentiation. The researchers suggested that these effects may be mediated by endocrine disruption, particularly through disruption of the estrogen signaling pathway. Another study by Romano et al. (2012) using in-vivo rat models showed that maternal exposure to glyphosate during pregnancy and lactation led

to alterations in the expression of genes involved in mammary gland development and function, as well as changes in milk production and composition.

Other studies on the contrary, including the U.S. Environmental Protection Agency (EPA) and the European Food Safety Authority's (EFSA), have concluded that glyphosate is unlikely to pose a carcinogenic risk to humans at the levels at which it is typically used.

According to the EPA, the safe levels of glyphosate exposure for humans are established by the reference dose (RfD), which is a measure of the maximum acceptable daily intake of a substance that does not pose a risk to human health. The EPA has set the RfD for glyphosate at 1.75 milligrams per kilogram of body weight per day (mg/kg/day) (EPA, 2020). This means that a person weighing 60 kilograms (approximately 132 pounds) could consume up to 105 milligrams of glyphosate per day without adverse effects.

However, there have been several studies that have suggested that people may be consuming glyphosate at levels that exceed the safe levels established by regulatory authorities. For example, one study analyzed the results of urine tests from 100 people living in urban areas in Germany and found that all the participants had detectable levels of glyphosate in their urine, and that the levels were higher in those who reported consuming conventionally grown food (Krüger et al., 2014). Another study showed that 83% of the breast milk samples tested positive for glyphosate, and 60% tested positive for glyphosate found in urine, with levels ranging from 0.20 to 17.0 ng/mL (Honeycutt and Rowlands, 2014).

The conflicting findings indicate that additional investigations and appropriate models are required to fully understand the potential health effects of glyphosate exposure. Nonetheless, the available evidence show that its role in breast cancer development is complex and may depend on a variety of factors (Muñoz et al., 2023; Stur et al., 2019; Thongprakaisang et al., 2013).

In light of the concerns about the potential links between glyphosate and breast cancer, we sought to evaluate the herbicide's short- and long-term effects on the differentiation and tumor initiation events of ductular and lobular mammary epithelial cells using 2D and an advanced three-dimensional (3D) culture models of non-tumorigenic mammary cells that are able to differentiate in culture.

In this study, we used ductal non-tumorigenic ER-negative HMT-3522 S1 human mammary epithelial cells, which form well-differentiated, growth-arrested, basally polarized structures or acini surrounding a lumen when cultured under 3D conditions. We also utilized mouse mammary ER-positive epithelial cells of the lobular type known as SCp2 cells, which express β-casein exclusively as a differentiation marker when grown in differentiation-promoting conditions with lactogenic hormones and basement membrane components. The objective of this investigation was to assess the influence of glyphosate on the onset of tumorigenesis by examining its effects on cellular proliferation and invasiveness in both ductal and lobular epithelial cells with distinct ER status. Additionally, differentiation patterns were assessed by examining apical polarity and lumen assembly in 3D culture of S1 cells, as well as the expression levels of β-casein in SCp2 cells grown in differentiation permissive conditions. We also used IPA software to predict pathways targeted by glyphosate and that are downstream of miR-183 overexpression.

Our data showed that glyphosate enhances invasion, disrupts acinar structure, and alters β-catenin localization in S1 cells, as well as increasing invasion and MMP-9 levels, downregulating β-casein, and upregulating miR-183 levels in SCp2 cell. Using IPA, we predict a pathway for the effect of glyphosate through the upregulation of miR-183 and consequently, downregulation of β-casein (CSN2), inhibition of mammary epithelial cell differentiation, and the onset of breast cancer. Consequently, we propose that glyphosate triggers tumor initiation events in nontumorigenic ductal and lobular mammary epithelial cells, mainly through the upregulation of the onco-miR, miR-183. Moreover, our study presents an opportunity to establish a framework for the implementation of preventive measures against breast cancer.

CHAPTER II

AIMS

Previous studies have shown that breast cancer is greatly attributed to lifestyle and environmental factors, which alter epigenetic regulations (Behravan et al., 2020; Orman et al., 2020). Given the acknowledged cancerous effects of glyphosate that were demonstrated along other risk factors on breast cancer formation (Duforestel et al., 2019; Thongprakaisang et al., 2013) in estrogen receptor (ER) positive and negative cell lines (Stur et al., 2019), we aim in this study to investigate the tumor initiation effects of glyphosate and its impact on differentiation using 2D and 3D mammary epithelial cell culture models of distinct ER status.

First, we aim to determine the effects of short-term and long-term glyphosate treatment on the growth rate, invasiveness, acinar formation, polarity proteins levels and localization in ER negative human-derived nontumorigenic S1 cells. We will also determine the effects of glyphosate treatments on the growth rate, invasiveness, MMP-9 levels, differentiation marker β-casein expression, and miR-183 levels in ER-positive mouse-derived SCp2 cells. Finally, we aim to identify possible pathways that are ER-dependent and ER-independent in SCp2 and S1 cells, respectively, using IPA data.

Taken together, our objective is to study the effect of glyphosate exposure on breast cancer initiation in non-tumorigenic mammary epithelial cells (SCp2 and HMT3522 S1), by investigating tumor initiation events such as invasiveness enhanced by MMP-9 release, the loss of apical polarity, as well as deregulation of the expression of miR-183 and β-casein, and the relocalization of β-catenin.

Therefore, our findings will provide insight on the effect of glyphosate on breast cancer initiation events and potentially contribute in part towards a policy for or against its use, in Lebanon and Globally.

CHAPTER III

LITERATURE REVIEW

A. The Mammary Gland

1. Structure and Stages of Development:

The mammary gland is a dynamic organ that undergoes significant changes during different stages of development and in response to hormonal signals. The glandular tissue is composed of epithelial cells, organised into ducts and lobules, surrounded by a layer of stromal cells, adipose tissue, and a network of blood and lymphatic vessels (Reviewed by Biswas et al., 2022). The mammary gland has two major kinds of epithelia: basal epithelium, which is primarily made up of myoepithelial cells, and luminal epithelium, which forms ducts and secretory alveoli (Runswick et al., 2001). Stromal cells provide support and regulate the function of the epithelial cells through the secretion of growth factors and cytokines, among other functions (Stadnyk, 1994; Schedin and Keely, 2011). The adipose tissue within the gland serves as an energy store for lactation, and the blood vessels provide nutrients and oxygen to the tissue, while the lymphatic vessels play a key role in immune surveillance and the removal of waste products. The efficient production and secretion of milk requires a balance of communication and coordination between the mammary epithelial cells, myoepithelial cells, and stromal cells, as well as various signaling pathways involving hormones, growth factors, and extracellular matrix molecules. Without this intricate interplay, the mammary gland may fail to function properly, leading to lactation problems or even breast diseases (Watson, 2006).

According to a review by Macias and Hinck (2012), the mammary gland develops during embryonic stages from an epithelial thickening called the mammary line, which runs along the ventral surface of the embryo. This thickening gives rise to the mammary bud, which eventually gives rise to the mature mammary gland. At this stage, the mammary gland is composed of a simple epithelial ductal tree, with no lobular differentiation. As the embryo develops, the ductal tree grows and branches, forming a complex network of ducts that extend towards the developing nipple. Puberty marks the beginning of mammary gland development in preparation for lactation. During puberty, the gland undergoes extensive branching morphogenesis, which involves the formation of ducts and the growth and differentiation of the glandular tissue (Brisken and O'Malley, 2010). This process is regulated by hormones such as estrogen, progesterone, and growth factors such as insulin-like growth factor (IGF) and epidermal growth factor (EGF) (Kleinberg et al., 2009). During pregnancy, the glandular tissue undergoes further differentiation, with the formation of alveoli and the secretion of milk components. This process is regulated by the hormones estrogen, progesterone, prolactin, and oxytocin (Howard & Gusterson, 2000). After lactation ceases, the gland undergoes a process of involution, which involves the remodeling and regression of the glandular tissue to its pre-pregnancy state. This process is triggered by the withdrawal of prolactin and involves the activation of apoptotic pathways (Watson, 2006; Green & Streuli, 2004; Humphreys & Hennighausen, 1999). Recent studies have advanced our understanding of mammary gland development. One study used lineage tracing to identify the progenitor cells that give rise to the mammary gland during embryonic development (Van

Keymeulen et al., 2011). The authors found that the mammary placode contains multiple types of progenitor cells, including basal and luminal progenitors, where basal stem cells are capable of generating all the different cell types within the mammary gland, whereas luminal stem cells are more restricted in their differentiation potential. Specifically, luminal stem cells are unable to give rise to myoepithelial cells. A recent study using a combination of single cell RNA sequencing and analysis of cells isolated from human milk identified transcriptional changes in the mammary gland during lactation (Twigger et al., 2022). The authors identified several genes involved in milk production and secretion that were upregulated in cells from the early lactation stage, including LALBA (encoding lactalbumin), CSN2 (encoding casein), and SLC2A1 (encoding glucose transporter 1). In contrast, cells from the late lactation stage exhibited increased expression of genes involved in immune regulation and tissue remodeling, including the immune-related genes CD74, HLA-DRB1, and CD14, and the tissue remodeling-related genes MMP11, CTGF, and THBS1. Xuan et al. (2022) shed light on the complex molecular mechanisms underlying the physiological process of mammary gland involution. It was shown that the mammary gland undergoes apoptosis and tissue remodeling, with upregulation of genes involved in apoptosis, autophagy, and extracellular matrix degradation. They revealed that several transcription factors such as ELF5, STAT5A, and STAT5B and non-coding RNAs such as miR-145, miR-200b, and lncRNA-TUG1, which are established to be implicated in epithelial-to-mesenchymal transition, were found to be differentially expressed during lactation and involution, suggesting their potential roles in regulating these processes. Another study used three-dimensional imaging techniques to visualise the structure of the mammary gland in unprecedented detail

(Ewald et al., 2008). The authors observed that mammary epithelial cells undergo collective migration guided by the ECM, with groups of cells moving in a coordinated fashion to form the growing branches of the mammary gland. It was also found that cell rearrangements occur during branching morphogenesis, with cells within the mammary epithelium actively rearranging their positions to facilitate the formation of new branches. These rearrangements involve both radial and planar cell intercalations, with cells moving both vertically and laterally to create the complex branching patterns seen in the mammary gland.

Recent studies have also investigated the role of stem cells in mammary gland development. Mammary gland stem cells are responsible for maintaining the mammary gland's homeostasis and are capable of differentiating into all the cell types of the mammary gland. A study by Dravis et al. (2015) showed that the transcription factor Sox10 plays a critical role in the maintenance of mammary gland stem cells. Using mouse models, the researchers demonstrated that Sox10 is necessary for the development of mammary stem/progenitor cells and for the maintenance of their self-renewal and differentiation capacity. They also showed that Sox10 is required for the development and maintenance of mesenchymal cells in the mammary gland.

In conclusion, the use of advanced techniques like single-cell sequencing, lineage tracing, and three-dimensional imaging has provided significant advancements in our understanding of the complexity of the mammary gland and its development. These new insights have important implications for the development of novel therapies aimed at addressing mammary gland diseases, particularly breast cancer. By gaining a better understanding of the molecular mechanisms underlying mammary gland development

and the pathogenesis of these diseases, we can develop targeted treatments that improve patient outcomes and reduce the morbidity associated with these diseases.

2. Epithelial Cell Differentiation Markers (Ductal vs. Lobular):

The mammary gland contains two types of epithelial cells: ductal and lobular epithelial cells. Ductal mammary epithelial cells (DMECs) line the ducts of the mammary glands and are responsible for the transport of milk components through the ducts to the nipple, while lobular epithelial cells are responsible for the synthesis and secretion of milk components (Reviewed by Biswas et al., 2022).

DMECs are polarized cells that play a critical role in the development and function of the mammary gland. Understanding the molecular mechanisms that control DMEC differentiation and function is important for the development of novel strategies for breast cancer prevention and treatment. As per the report by the American Cancer Society, invasive ductal carcinoma (IDC) represents a majority of breast cancer cases, accounting for around 75% of all diagnoses. Consequently, there has been a surge in research interest concerning the potential implications of cell polarity in the differentiation of ductal mammary epithelial cells (DMECs) in recent years. The establishment of polarity in cells mainly involves the coordinated action of three protein complexes that interact with each other and with the cytoskeletal and intercellular junction components, particularly for the apical-basal polarity. These complexes are known as Scribble, Crumbs, and Par, which define the basolateral, apical, and apical-lateral border domains, respectively. Several proteins within these complexes have been implicated in the development of cancer and are considered a novel type of tumor suppressor. (Assémat et al., 2008; Dow and Humbert, 2007; Etienne-Manneville and Hall, 2003b; Lee

and Vasioukhin, 2008). Apical polarity is characterized by the presence of tight junctions, which separate the apical and basolateral domains of the cell membrane and regulate the movement of molecules and ions between these compartments (Farquhar and Palade, 1963). Scribble has been shown to be necessary for the establishment of apical polarity and the differentiation of DMECs into luminal cells. In a study by Godde et al., it was found that loss of Scribble disrupted the expression of luminal differentiation markers and impaired the establishment of apical-basal polarity in DMECs. This shift in cell differentiation is mediated by activation of the MAPK/Fra1 signaling pathway, which is normally suppressed by Scribble (Godde et al., 2014). Several other polarity proteins have also been implicated in the regulation of MEC differentiation. For example, polarity proteins Patj and Lgl-1 have been shown to regulate the differentiation of DMECs into both luminal and myoepithelial cells (Kim et al., 2007; Grifoni et al., 2004).

Overall, the establishment of apical-basal and front-back polarity is critical for the differentiation of DMECs into luminal and myoepithelial cells, respectively. Polarity proteins, such as Crumbs, Scribble, Par3, Lgl1, and Patj, play essential roles in regulating these processes.

HMT-3522 S1 is a human mammary epithelial cell line that has been widely used to study mammary gland development, differentiation, and disease. S1 cells are a subpopulation of DMECs that have been shown to have stem cell properties and can differentiate into luminal and myoepithelial cells. 3D culture systems have been developed to better mimic the *in vivo* environment of the mammary gland (Petersen et al. 1992, Abbott, 2003; Lee et al., 2007) and study the differentiation of DMECs. Several studies have investigated the differentiation and polarity of HMT-3522 S1 cells using different

experimental approaches, including gene expression analysis, morphological changes, and functional assays. Petersen et al. (1992) investigated the role of cell polarity in HMT-3522 S1 cell differentiation. The authors found that the establishment of cell polarity, characterized by the formation of tight junctions and apical-basal polarity, is critical for HMT-3522 S1 cell differentiation. This study suggested that the establishment of cell polarity is an essential step in mammary gland differentiation and could be used as a marker of functional differentiation. Briand et al. (1987) used a combination of morphological analysis and functional assays to investigate the differentiation of HMT-3522 S1 cells. It was observed that differentiated cells displayed a flattened morphology and formed acinar structures, which are characteristic of mammary epithelial cells *in vivo*. This study demonstrated that HMT-3522 S1 cells undergo a functional differentiation process that mimics the differentiation of mammary epithelial cells *in vivo*. Overall, these studies suggest that HMT-3522 S1 cells are a useful model system for studying mammary gland development and differentiation.

Lobular mammary epithelial cells (LMECs) play a crucial role in breast development and function. LMECs are a heterogeneous population of cells that can differentiate into different cell types which is regulated by various molecular markers. Deugnier et al. (2006) used lineage tracing to investigate the differentiation of LMECs. The study found that LMECs are heterogeneous and can differentiate into different cell types, including luminal, myoepithelial, and basal cells. The authors show that LMECs may act as a reservoir of progenitor cells that can give rise to various cell types during breast development. Another study by Piggin et al. (2020) investigated the role of the transcription factor ELF5 in LMEC differentiation. It was found that ELF5 regulates the expression of various differentiation markers, including E-cadherin and cytokeratin 19. The

authors suggest that ELF5 plays a critical role in the differentiation of LMECs and may be a potential therapeutic target for breast cancer.

The differentiation marker beta casein has been extensively studied as a key protein that is expressed by differentiated mammary epithelial cells. Prolactin is a key regulator of beta casein expression in the mammary epithelial cell line (Doppler et al., 1989). When SCp2 cells are cultured in the presence of basement membrane (BM), two types of signals are generated. The first type involves physical signals such as cell rounding and clustering, while the second type involves β1-integrin-mediated biochemical signals from laminin. Upon activation by prolactin, these signals are required to induce phosphorylation of prolactin receptor and initiate downstream signaling cascade through the cell to the nucleus. This signaling cascade results in chromatin reorganization, which permits β-casein gene transcription, a marker of differentiation. These findings are supported by several studies (Xu et al., 2009; Alcaraz et al., 2008; Roskelley et al., 1994), suggesting the crucial role of BM and integrin-mediated signaling in regulating the expression of β-casein gene and mammary gland differentiation.

Thus, the expression of these markers during epithelial differentiation carries important implications in the context of breast cancer, given that neoplastic growth can originate from either ductal or lobular epithelial cells.

3. Cell-Cell Communication:

Cell-cell communication is essential for the proper functioning of multicellular organisms. This communication is mediated through cell junctions that facilitate the exchange of information between adjacent cells. Intercellular junctions connect epithelial

cells and play important roles in the maintenance of tissue morphology and homeostasis.

Nonetheless, given their overlapping localization and multiple interactions, it is becoming increasingly accepted that the constitutive proteins of these junctions form multi-junctional complexes which orchestrate the structure and function of cells. The five classes of cell-to-cell junctions include tight junctions (TJs), adherens junctions (AJs), desmosomes, gap junctions (GJs) and the recently described tunneling nanotubes (TNT).

Tight junctions are defined as areas of close contact between plasma membranes of adjacent cells, and are visualized as focal attachments between neighboring cell membranes that exclude the intercellular gap (Furuse, 2010). As previously reviewed (Balda & Matter 2008, Anderson & Van Itallie, 2009), the architecture of TJs is designed to serve as "permeability seals" that prevent solutes from escaping through the intercellular space. They achieve this by limiting lipid diffusion between the apical and basolateral domains and by controlling solute diffusion based on size and charge. Transmembrane claudins, occludins, tricellulin, Zona Occludens (ZO) family members, junctional adhesion molecules (JAMs), CRB-3, and blood vessel/epicardial substance (Bves) are some of the proteins that are related with TJs (Chiba et al. 2008, Wang and Margolis 2007, Brennan et al. 2010). Claudins command the TJs gate function and alter their conductivity (Krause et al. 2008), in addition to recruiting occludins to TJs (Martin and Jiang 2009). ZO-1 is one of several scaffold proteins found in the TJ cytoplasmic plaque. It is the first TJ protein to be found and is thought to act as a scaffold protein for transmembrane and cytoplasmic proteins, as well as a common partner for TJs, AJs, and GJs pro-

teins in their binding interactions (Utepbergenov et al. 2006; Giepmans 2004). TJ integrity is essential for lactogenesis. Although they are permeable in the mammary epithelia of the pregnant animal, TJs in the alveolar epithelium of lactating animals are highly impermeable to prevent milk leakage from the lumen. This is exhibited by the lower strands of TJ in pregnant animals as opposed to lactating ones (Morgan & Wooding, 1982). TJ strands are modified by hormones such as progesterone, prolactin, and glucocorticoids. It is well recognized that progesterone contributes to the development and persistence of pregnancy. Reduced progesterone levels at parturition cause significant mammary gland alterations that are crucial for the initiation of lactation. According to *in vivo* research, progesterone deprivation causes pregnant mice's mammary epithelia to close TJs (Stelwagen et al., 1998; Nguyen et al., 2001). In certain cancerous tissues, the expression of both claudins and occludins is decreased through various mechanisms, including epigenetic regulation, transcriptional control, and post-translational modification. Consequently, these tight junction proteins are trapped in the cytoplasm and are not transported to the membrane. This was shown in studies conducted by Lioni et al. (2007), Kominsky et al. (2003), and Ikenouchi et al. (2003). In breast cancer, ZO proteins that regulate proliferation can be suppressed (Hoover et al., 1998; Martin et al., 2004), and MAGIs, which are important in cell signaling, can be degraded by oncogenic viral proteins like the HTLV-1 Tax1 protein (Thomas et al., 2002; Ohashi et al., 2004). Occludins and claudin-1, -4, and -6 protein expression levels have been found to decline in breast cancer cell lines, whereas claudin-1 over-expression has been shown to increase apoptosis in tumor nodules of MDA-MB-361 breast cancer cells in three-dimensional (3D) cell culture (Osanai et al. 2007; Hoevel et al. 2004). In conclusion, the function of tight junctions extends beyond their traditional role of sealing the intercellular

space. Through acting as signaling hubs, they can influence the distribution of transcription regulators between nuclear compartments and tight junction complexes, as well as modulate chromatin-associated complexes. This expanded understanding of tight junctions provides new avenues for future research, as discussed in the review by Lelievre (2009).

Intercellular adhesion is mediated by AJs. They are made up of transmembrane proteins such as the well-established nectins and cadherins. It has been reported that nectins and cadherins, specifically E-cadherin, associate with p120-catenin and afadin, respectively (Takai et al. 2008). E-cadherin, N-cadherin, and P-cadherin are examples of classical cadherins that allow for strong cellular adhesion and cytoskeletal intermediate filament anchoring to the membrane (Lanigan et al. 2009). The Wnt signaling system, which is involved in cell proliferation, differentiation, gene transcription, and cell adhesion, is negatively regulated by E-cadherin. When E-cadherin binds to beta-catenin at the cell membrane, it sequesters beta-catenin and prevents it from translocating to the nucleus, where it acts as a transcriptional co-activator for genes that promote cell proliferation. This sequestration of beta-catenin by E-cadherin helps to regulate cell proliferation and maintain tissue integrity (Wijnhoven et al. 2000). Disrupting E-cadherin and P-cadherin function using antibody-soaked beads implanted in the mammary fat pad leads to disorganization of the mammary gland epithelium, affecting both the luminal and myoepithelial compartments. (Daniel et al., 1995; Reviewed by Lanigan et al., 2009). Furthermore, engineered mixtures of human luminal and myoepithelial cells self-organize into bilayers in culture, and this organization is disturbed by the addition of antibodies targeting E-cadherin or P-cadherin, providing additional evidence for the significance of differential cadherin expression in mammary epithelial development (Chanson

et al., 2011). Shamir et al. (2014) showed that loss of E-cadherin results in a switch from Twist1-mediated dissemination to a different mode of cancer cell movement that involves a loss of epithelial identity, further proving that epithelial polarity is disrupted by E-cadherin loss. As for the catenins, the loss of α-catenin lead to embryonic mortality due to disruption of the trophoblast epithelium. Additionally, Borcherding et al. (2018) used a proteomic approach to analyze protein expression levels in different types of cancer and found that the expression of E-cadherin and β-catenin varies significantly among different cancer types. The study also found that the loss of E-cadherin expression is strongly associated with the progression of breast cancer and that β-catenin expression is upregulated in many cancers, including breast cancer. Furthermore, the study explored the potential therapeutic implications of targeting E-cadherin and β-catenin in cancer treatment, suggesting that drugs that can stabilize or restore E-cadherin expression or inhibit β-catenin activity may have clinical benefits in certain cancer types.

β-catenin levels are often higher in the nuclei of tumor cells and linked to the activation of genes involved in cell cycle progression and proliferation, both of which are essential for the growth of tumors (Hatsell et al. 2003; Talhouk et al. 2013). In addition, β-catenin was found to contribute in apical polarity formation through its interaction with Connexin 43 in the mammary epithelium (Bazzoun at al. 2018).

Other transmembrane proteins from the six major cadherins subfamilies are found in desmosomal junctions (desmocollin and desmoglein) (Garrod and Chidgey 2008). Desmosomal proteins interact with other proteins like plakophilins, a family of catenin proteins, to recruit intermediate filaments to their localization (Nollet et al. 2000). These proteins engage heterophilically, unlike AJ-forming cadherins, to create strong junctions that hold cells together and place them in the appropriate location within the organ

(Runswick et al., 2001). Desmosomal cadherins have an intracellular domain, five calcium-binding extracellular domains, and are structurally identical to their classical counterparts. Desmosomes start out in the early embryo as small structures known as nascent desmosomes, which grow and develop into the larger and more complex desmosomes that are characteristic of adult tissues such as the heart and epidermis. It has been demonstrated that embryonic desmosomes are critical for maintaining the endoderm's mechanical integrity during a time when the embryo is beginning to diversify and take on shape and form (Gallicano et al., 1998).

Desmosomal cadherins are once again found commonly to be dysregulated in cancers. For instance, desmoglein-2 is either silenced or up-regulated in several cancers (Kurzen et al., 2003; Biedermann et al., 2005; Yashiro et al., 2006), while desmocollin-3 and desmocollin-2 are typically reported to be up-regulated in colorectal and breast cancers, respectively (Oshiro et al., 2005; Khan et al., 2006).

GJs are groups of intercellular channels that enable small molecules (<1.5 kDa) to diffuse directly between the cytoplasms of adjacent cells. Two connexons (also known as hemi-channels) that pair together to form GJ channels are made up of six trans-membrane proteins called connexins (Cxs). According to Sohl and Willecke (2004), the human Cx family has approximately 20 members, and the names of the different Cx proteins are frequently based on their molecular weights. Cxs with molecular weights of 43 kDa, 32 kDa, and 26 kDa, for example, are denoted as Cx43, Cx32, and Cx26, respectively. There are currently 21 identified connexins (Cxs) in humans. Cxs have been shown to associate with occludins, claudins, ZO-1 and ZO-2 (Herve et al., 2007; Tal-

houk et al., 2008). Cxs are found to be involved in luminal cell proliferation and differentiation, in proper production and ejection of milk, and in tumor suppression as reviewed by Banerjee, 2016.

Gap junctional-inducing complexes (GJICs), which represent a major conduit of second messengers, ions, and essential metabolites between adjacent cells (Kumar and Gilula, 1996), have been shown to play critical roles in various developmental and regulatory events, such as embryonic growth, bone modeling and neuronal signaling (Huettner et al., 2006; Houghton, 2005; Stains and Civitelli, 2005; Yang et al., 2007; Abraham et al. 2001; Naus and Bani-Yaghoub, 1998). It was demonstrated that GJIC induces partial differentiation of mammary epithelial cells in the absence of an exogenously supplied basement membrane, and that the formation of functional hetero-cellular GJs between epithelial and myo-epithelial cells correlates with mammary epithelial cell differentiation (El-Sabban et al., 2003; Talhouk et al., 2008). Moreover, inhibition of gap junctional intercellular communication (GJIC) in CID-9 mouse mammary cells cultured in the presence of exogenous basement membrane (under differentiation- permissive conditions) resulted in the downregulation of β-casein expression, a milk protein and a differentiation marker. Conversely, GJIC induction in the absence of basement membrane was sufficient to induce mammary epithelial differentiation, independent of ECM-induced STAT5 signaling (El-Sabban et al., 2003; Talhouk et al., 2011). Additionally, GJIC is linked to producing synchronized cardiac muscle contractions and maintaining tissue homeostasis, as well as the differentiation of a variety of tissues, including the mammary gland (Locke et al., 2000; Talhouk et al., 2005; Gong et al., 2007). Imbeault et al. (2009) showed that ECM-cell communication, specifically laminin, plays a role in the regulation of intrinsic Cx expression and function in postnatal

neural progenitor cells. It has been also demonstrated that Cx expression and GJ functionality are differently regulated at various phases of tumor development. For instance, Cx26 and Cx43 are shown to be upregulated during later stages of breast carcinomas (Jamieson et al., 1999), and correlated with increased metastatic potential, in primary mammary tumors (Naus et al., 1991; Soroceanu et al., 2001; Lee et al., 1992; Wilgenbus et al., 1992). In fact, Cx expression was shown to be up-regulated in lymph node metastases of breast cancer even when the primary tumor does not express Cxs (Kanczuga-Koda et al., 2006).

Cx43 particularly has been formerly shown to play both channel-dependent and channel-independent functions in mammary gland differentiation. The maintenance of apical polarity in the mammary epithelium is also regulated by Cx43. Cx43 silencing resulted in the loss of epithelial polarity in HMT3522 S1 cells cultured in 3D, which prepped the cells for cell cycle entry and a change in mitotic spindle orientation, thus indicating disruption of normal acinar morphology (Bazzoun at al., 2018). Ai et al. initially described the interaction of Cx43 with β-catenin within cardiac myocytes in 2000. Studies from our lab have additionally shown that Cx30, Cx32, and Cx43 associate with β -catenin, as well as with α -catenin, ZO-2, and ZO-1 in the mouse mammary epithelial SCp2 cell line. This interaction sequesters β-catenin away from the nucleus under differentiation- permissive conditions, and hence suggests that Cxs, in addition to their classical channel forming role, are involved in regulating intracellular signaling (Talhouk et al., 2008).

Both *in vitro* and *in vivo* studies support the tumor suppressive roles of Cxs in the mammary gland. Overexpression of Cx43 in MCF-7 and MDA-MB-231 cells reduced proliferation, cell cycle progression, and invasiveness independent of GJIC, suggesting

channel-independent mechanisms (Talhouk et al., 2013). Similarly, overexpression of Cx26 in MCF-7 and MDA-MB-435 cells suppressed proliferation, anchorage-independent growth, migration, and invasion, independent of GJIC (Kalra et al., 2006; Momiyama et al., 2003). Hirschi et al. (1996) and Qin et al. (2002) showed that overexpression of Cx26 or Cx43 in MDA-MB-231 and MDA-MB-435 cells suppressed xenograft tumor growth in nude mice. Ferrati et al. (2017) found that migration of MDA-MB-231 cells was impaired by exposure to Cx43-rich biovesicles extracted from plasma membranes of donor cells overexpressing functional Cx43-based GJs. Conditional mammary gland-specific knockout of Cx26 in mice predisposed the mammary gland to primary tumors (Stewart et al., 2015), and mice with heterozygous Cx43 mutation were more susceptible to mammary tumor lung metastasis (I Plante et al., 2011). Silencing Cx43 in Hs578T cells enhanced proliferation and anchorage-independent growth, associated with upregulation of VEGF and downregulation of TSP-1 (Shao et al., 2005). Recent studies showed that silencing Cx43 in HMT-3522 S1 cells induced mislocalization of membranous β-catenin, enhanced proliferation and cell cycle progression, and disrupted normal acinar morphology (Bazzoun et al., 2018). Cx43 expression was shown to revert the transformed phenotype of human glioblastoma tumor cells (Huang et al., 1998), U2OS osteosarcoma cell line and COS-7 cells (Zhang et al., 2003), in a GJIC-independent manner. Another study found that overexpressing Cx26 or Cx43 in three-dimensional (3D) cultures of MDA-MB-231 cells decreased growth rate and cell migration and caused partial re-differentiation of the organoids in a way that was independent of GJIC (McLachlan et al., 2006).

Taken together, the studies mentioned above demonstrate significant involvement of Cxs in the growth and malignancy of the mammary gland.

4. The Extracellular Matrix (ECM):

The local microenvironment has emerged as a major regulator almost two decades ago (Chua et al., 2010; Gouon-Evans et al., 2000; Keely et al., 1995; Koledova & Lu, 2017; O'Brien et al., 2012; Taddei et al., 2008; Woodward et al., 2001). Disruption of the mammary epithelial microenvironment is linked to breast cancer development (Insua-Rodriguez & Oskarsson, 2016; Majidinia & Yousefi, 2017; Soysal, Tzankov, & Muenst, 2015).

The ECM of the mammary gland is a dynamic and heterogeneous network that is regulated by several different factors, including hormonal cues, growth factors, and mechanical signaling. The ECM provides a scaffold for the epithelial cells and also regulates their behavior and function through the activation of signaling pathways (Keely et al., 1995). Alterations in the composition and organization of the ECM can lead to changes in tissue stiffness, cell adhesion, and matrix remodeling, which are all associated with breast cancer progression (Paszek et al., 2005). Targeting the ECM has emerged as a promising therapeutic strategy for breast cancer, with several drugs currently in clinical trials. However, the complexity and heterogeneity of the ECM also present significant challenges for the development of effective therapies (Reviewed by Zhao et al., 2021).

It was found that the ECM regulates the proliferation and differentiation of mammary epithelial cells and that disruptions in the ECM can lead to mammary gland dysfunction (Lu et al., 2012). This study also showed that the ECM is important for the proper organization of mammary epithelial cells into functional units called acini. The

ECM also plays an important role in breast cancer progression, by promoting the invasive behavior of breast cancer cells and that targeting specific ECM proteins can inhibit tumor growth and metastasis (Paszek et al., 2005; Lu et al., 2014). Armstrong et al. (2004) showed that type I collagen is highly expressed in breast tumors and is associated with increased cancer cell proliferation, migration, invasion, and resistance to chemotherapy. They also showed that blocking the interaction between type I collagen and cancer cells decreased tumor growth and metastasis in mouse models.

One of the key proteins present in the ECM is fibronectin, which plays a crucial role in regulating cellular behavior. A study by Schwarzbauer et al. (2011) demonstrated that fibronectin contributes to the formation of focal adhesions and regulates cellular contractility by activating integrins. This highlights the importance of the ECM in modulating cell behavior and suggests that alterations in ECM composition or structure may contribute to pathological conditions. Additionally, another study by Frantz et al. (2010) showed that the stiffness of the ECM also plays a critical role in cellular behavior. They demonstrated that a stiff ECM promotes the formation of focal adhesions and enhances cellular proliferation, whereas a soft ECM inhibits these processes. This emphasizes the importance of the physical properties of the ECM, particularly stiffness, in regulating cellular behavior.

B. Cellular Pathways that Contribute to Tumorigenicity

1. Loss of Polarity:

Cell polarity is the asymmetric distribution of cellular components in a cell, which is essential for its proper function. Loss of cell polarity has been implicated in several diseases, including cancer.

Loss of polarity can also lead to epithelial-to-mesenchymal transition (EMT), which is a key process in cancer metastasis. During EMT, epithelial cells lose their polarity and acquire a more mesenchymal phenotype, which allows them to migrate and invade surrounding tissues. The loss of polarity protein Crumbs has been shown to contribute to EMT and metastasis in breast cancer (Li et al., 2019). In this study, it was found that loss of Crumbs led to a disruption of cell polarity and increased expression of EMT markers, which promoted breast cancer metastasis.

Our recent study demonstrated that Cx43 is distributed in an apicolateral manner in the luminal epithelial cells of human breast tissue. Loss of Cx43 expression disrupts apical polarity and promotes cell multi-layering, which is a characteristic feature of tumor initiation in breast cancer (Fostok et al., 2019). Our findings suggest that individuals at a higher risk of developing breast cancer, such as obese patients, exhibit loss of Cx43 apical distribution and cell multi-layering in an inflammatory microenvironment (Deng et al., 2016). Our previous studies demonstrated that the silencing of Cx43 gene expression contributes to the formation of mammary tumors by promoting cell proliferation and progression through the cell cycle, and by causing the misplacement of membranous β-catenin. This leads to the loss of apical polarity, misalignment of the mitotic spindle, and the formation of multiple cell layers and loss of lumen, all of which are characteristic hallmarks of tumor initiation. Furthermore, silencing Cx43 also activates signaling pathways that encourage invasion in non-tumorigenic breast epithelium (Bazzoun et al., 2019; Fostok et al., 2019). Similarly, Lesko et al. (2015) demonstrated that the disturbance of epithelial polarity served as an indicator for the onset of tumors derived from epithelial tissues. The findings highlight the importance of APC tumor

suppressor in regulating epithelial cell polarization and provide insights into the mechanisms underlying tumor initiation and progression. In fact, one of the early changes observed in breast tissues of women at higher risk of developing breast cancer is the loss of polarity, which is characterized by the disturbance of the bilayered epithelial architecture and the accumulation of cells towards the lumen (Van de Vijver and Peterse 2003).

One important factor that can contribute to the loss of polarity in MECs is genetic mutations. Several genes have been identified that are important for establishing and maintaining polarity in mammary epithelial cells (MECs). For example, the tumor suppressor gene PTEN is frequently mutated in breast cancer, and its loss has been shown to disrupt the polarity of MECs (Kechagioglou et al., 2014). In addition, mutations in the genes encoding for polarity proteins such as Scribble, Par3, and Crumbs can also lead to the loss of polarity in MECs and contribute to tumorigenesis (Martin-Belmonte & Mostov, 2008). Another factor that can contribute to the loss of polarity in MECs is extracellular signals. The extracellular matrix (ECM) plays an important role in regulating the polarity of MECs. The ECM can provide both structural support and signaling cues that can influence the polarity of MECs. β1-integrins, laminin-111, and dystroglycan are key factors involved in establishing polarity, as demonstrated in 3D cultures of mammary and Madin-Darby canine kidney (MDCK) cells (O'Brien et al., 2001; Weir et al., 2006; Yu et al., 2005). These integrins guide the orientation of luminal cells within the alveoli, allowing for the formation of fluid-filled cavities that support the vectorial secretion and intake of molecules. However, the signaling mechanisms through which integrins regulate the establishment of the apical surface are not yet fully understood (O'Brien et al., 2002), despite their interaction with the extracellular matrix.

In addition to genetic mutations and extracellular signals, intracellular signaling pathways can also contribute to the loss of polarity in MECs. One example is the Wnt signaling pathway, which is frequently activated in breast cancer. The Wnt signaling pathway has been shown to regulate the polarity of MECs through its effects on the protein β-catenin (Reviewed by Patel et al., 2019; Debnath et al., 2003).

In conclusion, the loss of polarity, driven by dysregulation of polarity proteins such as connexin 43 and beta-catenin, is a critical factor that contributes to tumorigenesis. Understanding the mechanisms underlying the dysregulation of these proteins and their role in maintaining cellular polarity may provide insights into novel therapeutic approaches for cancer treatment.

2. *Loss of Cell Cycle Control:*

Mitosis is not only essential for the segregation of chromosomes but can also direct tissue architecture and cell fate. In general, astral microtubules position and orient the entire mitotic spindle by rotating it into the defined orientation relative to the cell axis (Giansanti et al. 2001; O'Connell and Wang 2000). The cell cycle is a tightly regulated process that controls cell growth and division. It is essential for normal cellular function and tissue homeostasis (Shakelford et al., 1999). Alterations in the cell cycle machinery can result in uncontrolled cell proliferation, which is a hallmark of cancer. Loss of cell cycle control has been implicated in the development and progression of many types of cancer (Reviewed by Mercadante & Kasi, 2022). One of the key regulators of the cell cycle is the tumor suppressor protein p53. p53 plays a critical role in maintaining genomic stability by inducing cell cycle arrest or apoptosis in response to DNA damage or other cellular stresses. Mutations in the p53 gene are common in many types of cancer,

and loss of p53 function is often associated with poor prognosis (Huszno & Grzybowska, 2018; Leroy et al., 2002). In addition to p53, other cell cycle regulators such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) also play important roles in cell cycle control. Loss of cell cycle control can occur through various mechanisms, including mutations in cell cycle regulators, epigenetic alterations, and dysregulation of signaling pathways (Ma et al., 2015). For example, in breast cancer, loss of the CDK inhibitor p16INK4a has been shown to be a frequent event, and its loss has been associated with poor prognosis (Witkiewicz et al., 2011). In addition, dysregulation of signaling pathways such as the PI3K/Akt/mTOR pathway can also lead to loss of cell cycle control and contribute to tumorigenesis (Paplomata & O'Regan, 2014). Studies have shown that the depletion of certain proteins, including Par6, Par3, Cdc42, aPKC, or Dlg, can cause defects in tissue architecture due to misorientation of cellular divisions. The interaction between Dlg and LGN, an adaptor protein rich in Leu-Gly-Asn repeats, mediates the communication between cell polarity and the direction of cell divisions. Dlg binds LGN to the lateral side of the plasma membrane, where LGN interacts with the heterotrimeric G-protein, Gαi. During mitosis, LGN partners with NuMA, a nuclear mitotic apparatus protein that recognizes astral microtubules and dynein. To ensure proper segregation of mitotic chromosomes, the NuMA/LGN complex must localize to the cell midcortex, which requires the activation of aPKC. The Par3/Cdc42-GTP complex activates aPKC at the apical surface, which prevents the NuMA/LGN complex from localizing to the apical surface and thus inhibits vertical alignment of the spindle. (Figure 5B). These findings have been reported in various studies, including Hao et al. (2010), Jaffe et al. (2008), Zheng et al. (2010), Bergstralh et al. (2013), Durgan et al. (2011), and Du and Macara (2004).

Another study by Beroukhim et al. (2010) analyzed genomic alterations in a large cohort of cancer patients and identified frequent mutations in cell cycle regulators such as TP53, CDKN2A, and RB1. The study also identified genetic alterations in other genes that regulate the cell cycle, including cyclins and CDKs, further highlighting the importance of cell cycle control in tumorigenesis.

In addition to the above examples, loss of cell cycle control has been implicated in the development of many other types of cancer with recent ongoing research, including pancreatic cancer (Yao et al., 2022), prostate cancer (Ben-Salem et al., 2021), and ovarian cancer (Li et al., 2021), among others.

C. Breast Cancer Classification and Stages

Breast cancer is the most common cancer in women worldwide and is the second leading cause of cancer-related deaths among women (American Cancer Society, 2023). The classification and staging of breast cancer play a crucial role in determining the prognosis and developing appropriate treatment strategies.

According to the World Health Organization (WHO) classification, breast cancer can be classified based on different factors, including histological features, molecular characteristics, and clinical features.

First, histological classification is based on the microscopic appearance of breast cancer cells and their organization. The most common types of breast cancer based on histological classification are ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), and others.

DCIS is a non-invasive breast cancer that arises from the lining of the milk ducts and has not spread beyond the ducts. It is often detected through mammography and has an

excellent prognosis. A study by Kerlikowske (2010) found that DCIS accounts for approximately 20% of all breast cancers, and the incidence has been increasing over the past few decades due to the widespread use of screening mammography. IDC on the other hand is the most common type of invasive breast cancer, accounting for approximately 80% of all cases. It arises from the milk ducts and invades the surrounding breast tissue. IDC can also spread to other parts of the body, such as the lymph nodes, and is associated with a higher risk of recurrence and mortality. ILC is a less common type of invasive breast cancer, accounting for approximately 10-15% of all cases (Sultan et al., 2019). It arises from the milk-producing lobules and can invade the surrounding breast tissue and other parts of the body. ILC is associated with a unique gene expression profile compared to IDC and has distinct molecular features that may impact treatment response (Ciriello et al., 2015).

Additionally, molecular classification is based on the molecular characteristics of breast cancer cells, such as the expression of hormone receptors (HR), human epidermal growth factor receptor 2 (HER2), and gene expression profiling. Molecular classification has been instrumental in developing personalized treatment strategies for breast cancer.

HR-positive breast cancer accounts for approximately 60-70% of all breast cancers and is characterized by the presence of estrogen receptor (ER) and/or progesterone receptor (PR) on the surface of cancer cells (National Cancer Institute, Hormone Therapy for Breast Cancer, 2012). HR-positive breast cancer is often treated with endocrine therapy, such as tamoxifen or aromatase inhibitors, which block the production or activity of estrogen. As reviewed by Bhatia et al. (2019), HR-positive breast cancer has a better prognosis compared to HR-negative breast cancer.

HER2-positive breast cancer accounts for approximately 15-20% of all breast cancers and is characterized by the overexpression of HER2 on the surface of cancer cells. HER2-positive breast cancer is often treated with targeted therapy, such as trastuzumab or pertuzumab, which block the activity of HER2.

Gene expression profiling has identified different subtypes of breast cancer based on their gene expression patterns (Perou et al., 2000). The most common subtypes of breast cancer based on gene expression profiling are luminal A, luminal B, HER2-enriched, and basal-like.

Luminal A breast cancer is characterized by the expression of ER, PR, and low levels of HER2. It accounts for approximately 40-50% of all breast cancers and is associated with a better prognosis compared to other subtypes. Luminal A breast cancer is often treated with endocrine therapy, such as tamoxifen or aromatase inhibitors.

Luminal B breast cancer is also characterized by the expression of ER, but has higher levels of HER2 and/or Ki-67, a marker of cell proliferation (Sorlie et al., 2001). It accounts for approximately 10-20% of all breast cancers and is associated with a worse prognosis compared to luminal A breast cancer. Luminal B breast cancer is often treated with a combination of endocrine therapy and chemotherapy. HER2-enriched breast cancer is characterized by the overexpression of HER2 and low levels of ER and PR. It accounts for approximately 10-15% of all breast cancers and is associated with a worse prognosis compared to luminal A breast cancer. HER2-enriched breast cancer is often treated with HER2-targeted therapy, such as trastuzumab or pertuzumab, in combination with chemotherapy (Swain et al., 2015).

Basal-like breast cancer is characterized by the absence of ER, PR, and HER2, and the expression of basal markers, such as cytokeratin 5/6 and epidermal growth factor receptor (EGFR) (Perou et al., 2000). It accounts for approximately 10-15% of all breast cancers and is associated with a worse prognosis compared to other subtypes. Basal-like breast cancer is often treated with chemotherapy, as there are currently no targeted therapies available. Bernardo et al. (2013) found that FOXA1 expression is negatively correlated with the basal-like phenotype in breast cancer cells, and that overexpression of FOXA1 in basal-like breast cancer cells leads to a shift towards a luminal phenotype, characterized by increased expression of luminal markers and decreased expression of basal markers, suggesting that targeting FOXA1 may be a potential therapeutic strategy for this subtype of breast cancer. The majority of triple negative breast cancers are carcinomas with low differentiation that exhibit high aggressiveness, frequent local recurrence, and metastases to other organs. These cases are prevalent in younger women and often linked to pathogenic mutations in the BRCA1 gene, and in rare instances, the BRCA2 gene, which result in the development of hereditary forms of breast cancer (Mehrgou et al., 2016).

Clinical classification is based on the stage of breast cancer, which is determined by the size and extent of the tumor and whether it has spread to the lymph nodes or other parts of the body (American Joint Committee on Cancer (AJCC), 1977). The stage of breast cancer is a crucial factor in determining the prognosis and developing appropriate treatment strategies.

The staging of breast cancer is based on the TNM system (Denoix, P., 1952, AJCC, 1977), which stands for tumor size and extent (T), lymph node involvement (N), and

distant metastasis (M). The TNM system is used to determine the stage of breast cancer, which ranges from stage 0 (DCIS) to stage IV (metastatic breast cancer).

Stage 0: DCIS is a non-invasive breast cancer that has not spread beyond the ducts.

Stage I: The tumor is less than 2 cm in size and has not spread to the lymph nodes or other parts of the body.

Stage II: The tumor is between 2-5 cm in size and may have spread to nearby lymph nodes, but not to other parts of the body.

Stage III: The tumor is larger than 5 cm in size and may have spread to nearby lymph nodes or tissues, but not to other parts of the body.

Stage IV: The cancer has spread to other parts of the body, such as the bones, liver, or lungs (National Cancer Institute).

D. Epigenetics in Breast Cancer

According to the International Agency for Research on Cancer (IARC), over 2.3 million new cases of breast cancer were diagnosed in 2020 alone.

Epigenetic changes have been increasingly recognized as one of the hallmarks of cancer development and progression (Hanahan and Weinberg, 2000). Epigenetic modifications are changes in gene expression that are heritable but not caused by alterations in the DNA sequence. DNA methylation, histone modifications, and non-coding RNAs are the most commonly studied epigenetic alterations in cancer.

DNA methylation is a crucial epigenetic modification that regulates gene expression by adding a methyl group to the cytosine residues in CpG dinucleotides (Feinberg and Vogelstein, 1983). Several studies have shown that global DNA hypomethylation and hypermethylation of specific genes are common in breast cancer cells (Shen et al., 2020;

Zhou et al., 2018; Bae et al., 2004; Esteller et al., 2001). Yang Gao (2018) found that DNA methylation patterns in normal breast tissue were more strongly associated with breast cancer status than copy-number variants (CNVs). The study analyzed DNA samples from 44 breast cancer patients and 44 healthy individuals and identified differentially methylated regions (DMRs) and CNVs using high-density arrays. The researchers found that DMRs in normal tissue had a higher predictive value for breast cancer status than CNVs, and that specific DMRs were associated with specific breast cancer subtypes. The study suggests that DNA methylation patterns in normal tissue may serve as potential biomarkers for breast cancer risk and early detection. Ming Qi and Xiang Xiong (2018) conducted a meta-analysis to investigate the association between promoter hypermethylation of five genes (RARβ2, DAPK, hMLH1, p14, and p15) and progression of breast cancer. The results of the meta-analysis showed that promoter hypermethylation of all five genes was significantly associated with progression of breast cancer. Moreover, the degree of promoter hypermethylation was positively correlated with tumor stage, lymph node metastasis, and poor overall survival. The study suggests that promoter hypermethylation of these genes may serve as potential biomarkers for the early detection and prognosis of breast cancer, and may provide insights into the underlying mechanisms of breast cancer progression. Another study conducted by Asiaf et al. (2019) investigated the relationship between protein expression and methylation of the DAPK1 gene with clinicopathological features in invasive ductal carcinoma patients from Kashmir. It was found that methylation of the DAPK1 promoter region was found to be higher in breast cancer tissues than in adjacent normal tissues and was associated

with decreased protein expression of DAPK1. The study suggests that DAPK1 methylation may be a potential biomarker for breast cancer prognosis and may contribute to breast cancer progression by regulating apoptosis and cell cycle progression.

Histone modifications are another important epigenetic alteration that regulates gene expression by adding or removing chemical groups to the histone proteins that make up the chromatin structure. Histone modifications can either activate or repress gene expression. As reviewed by Li et al. (2013), aberrant histone modifications have been found to be associated with breast cancer development and progression.

One study conducted by Lu et al. (2010) found that the histone methyltransferase EZH2 was overexpressed in breast cancer tissues compared to normal breast tissues. The researchers also found that EZH2 overexpression was associated with poor prognosis in breast cancer patients. EZH2 overexpression was also found to be associated with increased histone H3 lysine 27 trimethylation (H3K27me3) levels, a repressive histone modification. Therefore, targeting EZH2 may be a promising therapeutic strategy for inhibiting tumor angiogenesis and improving breast cancer outcomes.

Another study conducted by Shan et al (2017) reported on the role of HDAC2 (histone deacetylase 2) in breast cancer. The authors found that HDAC2 is overexpressed in breast cancer cells and tissues, and this overexpression is associated with aggressive clinicopathological features such as higher tumor grade, larger tumor size, and positive lymph node status.

Furthermore, the study found that HDAC2 overexpression is linked to activation of the DNA-damage response pathway, which is a cellular mechanism that responds to DNA damage and prevents the formation of cancerous cells. The authors suggest that

HDAC2 may contribute to breast cancer progression by interfering with the DNA-damage response pathway, leading to the accumulation of DNA damage and promoting the development of cancerous cells.

Non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are emerging as critical epigenetic regulators of gene expression. Dysregulation of non-coding RNAs has been found to be associated with breast cancer development and progression.

Jin et al (2017) investigated the role of the long non-coding RNA (lncRNA) MALAT1 in promoting cell proliferation and metastasis in epithelial ovarian cancer via the PI3K-AKT pathway. The authors found that MALAT1 was upregulated in epithelial ovarian cancer tissues compared to adjacent normal tissues, and that high levels of MALAT1 expression were associated with advanced tumor stage and poor prognosis.

Further experiments in ovarian cancer cell lines showed that MALAT1 promoted cell proliferation and invasion by activating the PI3K-AKT signaling pathway. In addition, MALAT1 was found to regulate the expression of several genes involved in cell proliferation, apoptosis, and invasion, including Bcl-2, Cyclin D1, MMP-2, and MMP-9. Si et al. (2019) looked into the role of the lncRNA H19 in regulating cell growth and metastasis in breast cancer via miR-138. It was found that H19 was upregulated in breast cancer tissues compared to adjacent normal tissues, and that high levels of H19 expression were associated with poor prognosis.

MicroRNAs (miRNAs) are small non-coding RNA molecules that have been implicated in the development and progression of breast cancer. Studies have reported increased levels of specific circulating microRNAs (miRNAs) in breast cancer patients with advanced disease. For example, miR-10b, miR-34a, and miR-155 were found to be

elevated in metastatic breast cancer (mBC) patients (Roth et al., 2010), while miR-10b and miR-373 (Chen et al., 2013), as well as miR-20a and miR-214 (Schwarzenbach et al., 2012), were upregulated in patients with lymph node positive breast cancer compared to those without lymph node involvement. MiR-10b has been suggested as a potential biomarker for mBC metastasis to the brain and bones (Ahmad et al., 2014), and miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801 were found to be significantly upregulated in plasma of mBC patients with circulating tumor cells (Madhavan et al., 2012). Upregulation of miR-105 was shown to predict metastasis in early onset breast cancer (Zhou et al., 2014), while elevated levels of miR-17 and miR-155 could discriminate metastatic from non-metastatic breast cancers (Eichelser et al., 2013).

Several studies have investigated the role of miR-183 in breast cancer. Li et al. (2014) found that the miR-183/-96/-182 cluster was upregulated in most breast cancers and that its overexpression increased cell proliferation and migration in breast cancer cell lines. The researchers also identified that the cluster targets and downregulates the expression of several tumor suppressor genes, including EGR1, ITGA9, and TIMP2, which are involved in the regulation of cancer cell proliferation and migration. The study suggested that the miR-183/-96/-182 cluster may play a crucial role in breast cancer progression and could be a potential therapeutic target for breast cancer treatment. Moreover, Li et al. (2020) aimed to investigate the role of miR-183-5p in breast cancer progression and its interaction with Four and a Half LIM Protein 1 (FHL1). The researchers found that miR-183-5p was significantly upregulated in breast cancer tissues and cell lines, and its overexpression promoted cell proliferation, migration, invasion,

and angiogenesis *in vitro*. The overexpression of miR-183-5p also reduced FHL1 expression levels by directly targeting its 3'-untranslated region (3'UTR). Moreover, the knockdown of FHL1 expression by siRNA could mimic the effects of miR-183-5p overexpression on breast cancer cells. The study suggests that miR-183-5p could promote breast cancer progression by downregulating FHL1 expression, which may have implications for breast cancer diagnosis and treatment (Li et al., 2020). In another study, Song et al. (2016) investigated the potential of the miR-183/182/96 cluster as a prognostic biomarker for breast cancer. They analyzed the expression of these miRNAs in breast cancer tissues and found that their high expression was associated with advanced stages of breast cancer, lymph node involvement, and poor prognosis. Furthermore, the authors demonstrated that the miR-183/182/96 cluster promotes breast cancer cell proliferation, migration, and invasion, suggesting that it may contribute to the progression of the disease. Additionally, Naser Al Deen et al. (2022) showed that over-expression of miR-183-5p or miR-492 was found to induce pre-neoplastic phenotypes similar to those reported upon Cx43 loss, such as cell multi-layering, lack of lumen formation, and enhanced invasion (Bazzoun. et al., 2019; Fostok et al., 2019), and may act as oncomiRs and possible biomarkers of increased breast cancer risk (Naser Al Deen et al., 2022).

Epigenetic alterations are reversible and can be targeted by epigenetic therapy. Epigenetic therapies aim at restoring normal gene expression patterns in cancer cells by inhibiting or activating specific epigenetic modifiers. Several epigenetic therapies have been developed for breast cancer treatment, including DNA methyltransferase inhibitors, histone deacetylase inhibitors, and bromodomain and extra-terminal domain (BET) inhibitors (Shi et al., 2015; Fenaux et al., 2009; Silverman and Mufti, 2005).

The review article by Garcia-Martinez et al. (2021) discussed the role of epigenetic mechanisms in breast cancer therapy and resistance. The authors focused on the current state of epigenetic-based therapies for breast cancer and noted that these therapies have shown promise in preclinical studies, but their efficacy in clinical trials is still limited, partly due to the development of drug resistance. They also highlighted the importance of identifying biomarkers of epigenetic modifications and drug resistance to develop personalized therapies for breast cancer. Additionally, for women receiving medical care for breast cancer, modifying their lifestyle can diminish the likelihood of recurrence and amplify the prospects of survival (Pieta et al., 2012). It is therefore crucial to educate women about the significance of lifestyle modifications in preventing breast cancer and enhancing the survival rate and recurrence risk in breast cancer patients.

E. Factors that Contribute to Tumorigenicity

1. Environmental/Lifestyle Contaminants:

Environmental factors are recognized to contribute significantly to the development of breast cancer. Studies have shown that environmental factors such as exposure to chemicals, radiation, and lifestyle factors like diet and physical activity play a significant role in the development of breast cancer (Chen et al., 2011; Brody et al., 2007; Reynolds et al., 2004; Boyd et al., 2003).

Studies have reported that a high-fat diet is associated with an increased risk of breast cancer. A study conducted by World Cancer Research Fund/American Institute for Cancer Research (2018) reported that high-fat diets were linked to an increased risk of postmenopausal breast cancer. A study by Aune et al. (2017) showed that consuming a diet rich in fruits and vegetables was associated with a reduced risk of breast cancer in

both pre- and postmenopausal women. Additionally, physical activity has been linked to a reduced risk of breast cancer. A study by Friedenreich et al. (2010) reported that women who engaged in regular physical activity had a reduced risk of breast cancer compared to women who were physically inactive. The study also found that the protective effect of physical activity was more significant for postmenopausal breast cancer. Alcohol consumption has been identified as a risk factor for the development of breast cancer. A study by the World Cancer Research Fund/American Institute for Cancer Research (2018) reported that alcohol consumption was linked to an increased risk of breast cancer in women. The study found that the risk of breast cancer increased with the amount of alcohol consumed. Alcohol consumption has also been identified as a risk factor for the development of breast cancer. A study by the World Cancer Research Fund/American Institute for Cancer Research (2018) reported that alcohol consumption was linked to an increased risk of breast cancer in women. The study found that the risk of breast cancer increased with the amount of alcohol consumed.

Exposure to ionizing radiation, a type of radiation that has enough energy to remove tightly bound electrons from atoms and causing damage to DNA, has also been associated with an increased risk of breast cancer (Boyce et al., 1991; Pierce & Preston, 2000; Land et al., 2003; Ronckers et al., 2008). For example, a study by Boice et al. (2012) reported that women who were exposed to ionizing radiation during childhood had an increased risk of breast cancer. The risk was highest for women who received the highest radiation doses. Another study investigated the role of the long non-coding RNA GAS5 in regulating the response of breast cancer cells to ionizing radiation. The authors found that GAS5 expression was significantly reduced in breast cancer cells compared to normal breast cells. Moreover, breast cancer cells with low GAS5 expression were more

resistant to ionizing radiation, whereas those with high GAS5 expression were more sensitive to radiation (Ma et al., 2022).

Importantly, chemical exposure has been associated with the development of breast cancer, including hormone-disrupting chemicals (Brody et al., 2007; Diamanti-Kandarakis et al., 2009; White et al., 2016). Endocrine-disrupting chemicals (EDCs) are compounds that interfere with the body's hormone systems, leading to adverse health outcomes, including breast cancer. EDCs have been identified in several environmental pollutants, including pesticides, industrial chemicals, and plastics. A study conducted by Martin et al. (2018) reported that exposure to polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), and phthalates was linked to an increased risk of breast cancer. Moreover, Jayaseelan et al. (2021) reviewed existing literature on the subject and identified several potential mechanisms by which DDT exposure could contribute to breast cancer development. These include DDT's ability to mimic estrogen and bind to estrogen receptors, as well as its effects on DNA methylation and alterations to gene expression. The authors also recommend that efforts be made to limit exposure to DDT and other environmental toxins in order to reduce the risk of breast cancer and other diseases.

Pesticides are chemicals used to control pests that attack crops or livestock. Studies have identified several pesticides, including organochlorines, organophosphates, and carbamates, as potential risk factors for breast cancer. While the evidence linking pesticides to breast cancer is not conclusive, it is important to be aware of the potential risks associated with exposure to these chemicals. For example, Kaur et al. (2019) found that exposure to organochlorine pesticides is a significant risk factor for developing breast cancer in young Indian women. Blood samples were collected from 156 young women

diagnosed with breast cancer and tested for levels of six commonly used organochlorine pesticides. The results showed that women with breast cancer had significantly higher levels of all six pesticides compared to the control group.

The study also found that the risk of breast cancer increased with increasing levels of organochlorine pesticides, and the risk was highest for women with the highest levels of these pesticides in their blood.

Studies have reported that exposure to industrial chemicals, including bisphenol A (BPA), is associated with an increased risk of breast cancer. BPA is a chemical used in the manufacture of plastics and is commonly found in food packaging, water bottles, and canned foods. For instance, Sengupta et al (2013) investigated the potential role of bisphenol and bisphenol A in promoting the growth and survival of breast cancer cells through their interaction with the estrogen receptor alpha (ERα). The study found that both bisphenol and bisphenol A stimulated the proliferation of ERα-positive breast cancer cells and inhibited their apoptosis (programmed cell death). This effect was mediated by the activation of the ERα signaling pathway and the upregulation of downstream genes involved in cell growth and survival. The authors also demonstrated that both chemicals enhanced the binding of ERα to estrogen response elements (EREs) in the DNA, suggesting that these chemicals can mimic the effects of estrogen in breast cancer cells. In another study, Dong et al (2011) investigated the mechanism by which bisphenol A (BPA), a common environmental chemical, stimulates the growth of breast cancer cells. The study found that BPA rapidly activated the extracellular signal-regulated kinase 1/2 (Erk1/2) signaling pathway in human breast cancer cells, leading to increased cell proliferation and survival.

While the exact mechanisms by which these environmental and lifestyle factors contribute to breast cancer are not fully understood, it is important to continue researching these factors to better understand how they contribute to the development of breast cancer. Further research may help to identify new prevention strategies and improve the early detection and treatment of breast cancer.

2. *Glyphosate: Environmental Contaminant:*

Glyphosate is a widely used herbicide that was first introduced by Monsanto in 1974 under the trade name Roundup. It is commonly used in agriculture, forestry, and home gardening to control weeds. Glyphosate works by inhibiting the activity of an enzyme called EPSP synthase, which is required for the production of certain amino acids in plants (Franz, 1977). This results in the death of the treated plants. Christelle Bou-Mitri et al. (2022) investigated the presence and exposure of glyphosate in bread and flour products in Lebanon. The researchers collected and analyzed 98 samples from various regions in Lebanon and found that glyphosate was present in all samples, with 39% of samples exceeding the maximum residue limit set by the European Union. The study suggests that there is a potential health risk associated with glyphosate exposure through bread and flour products in Lebanon and highlights the need for stricter regulations and monitoring of glyphosate use in agriculture. In 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as a "probable human carcinogen" based on studies in animals and limited evidence in humans. However, this classification has been widely debated, with many studies and organizations disputing the IARC's findings. For example, in 2017, the European Chemicals Agency (ECHA) concluded that

glyphosate is not carcinogenic. One study included 21 case-control studies and two cohort studies, with a total of 502,537 participants to study the risk of non-Hodgkin lymphoma (NHL) and multiple myeloma (MM) upon exposure to glyphosate. The findings suggest that exposure to glyphosate is associated with an increased risk of NHL and MM, particularly in North America (Donato et al., 2020). Stur et al. (2019) investigated the effects of glyphosate-based herbicides (GBHs) at low doses on canonical pathways in estrogen receptor-positive (ER+) and estrogen receptor-negative (ER-) breast cancer cell lines. The study found that GBHs at low doses (10^{-12} M to 10^{-6} M) significantly affected several canonical pathways in ER+ and ER- breast cancer cell lines, which included those related to hormone signaling, cell cycle regulation, DNA damage response, and apoptosis. Moreover, the effects of GBHs on these pathways were more pronounced in ER- cell lines, and the effects were dose-dependent, with higher doses resulting in greater effects (Stur et al., 2019). Thongprakaisang et al. (2013) found that glyphosate can promote the growth of estrogen-dependent human breast cancer cells. This study suggests that glyphosate exerts its effects on breast cancer cells by acting as an estrogen mimetic and activating ER-mediated signaling pathways. Moreover, Glyphosate also increased the expression of genes involved in cell proliferation and reduced the expression of genes involved in cell cycle arrest and apoptosis.

In another study, Zhang et al. (2019) examined the association between glyphosate exposure and breast cancer risk in a large cohort of women. The study found that women who had the highest levels of glyphosate in their urine had a significantly increased risk of developing breast cancer compared to women with lower levels of glyphosate. In contrast, Andreotti et al. (2018) investigated the association between

glyphosate exposure and cancer risk in the Agricultural Health Study cohort, which included over 54,000 pesticide applicators. The study found a positive association between glyphosate exposure and risk of non-Hodgkin lymphoma, but no association with breast cancer was observed. More recently, Muñoz et al. (2023) found that glyphosate can mimic the effects of the hormone 17β-estradiol in breast cancer cells, thereby promoting the activity of estrogen receptor alpha (ERα). The researchers showed that glyphosate increased the growth of breast cancer cells, even at low doses (1×10^{-8} to 1×10^{-3} M), by promoting the activation of ERα. The study also found that glyphosate promoted the migration of breast cancer cells, suggesting that it may contribute to breast cancer metastasis.

One of the studies aiming at investigating the potential mechanism underlying the link between glyphosate exposure and breast cancer development found that glyphosate exposure altered the DNA methylation patterns of mammary epithelial cells, leading to the reprogramming of the cells' epigenome in a TET3-dependent manner (Duforestel et al., 2019). The altered epigenetic landscape resulted in the upregulation of oncogenes and the downregulation of tumor suppressor genes, which may contribute to the development of breast cancer. The study also found that the effects of glyphosate exposure were more pronounced in cells that had undergone a previous transformation triggered by the upregulation of miR-182-5p, suggesting that glyphosate may enhance the tumorigenic potential of already-transformed cells.

In summary, the potential link between glyphosate exposure and breast cancer risk remains a controversial topic. While some studies have suggested a positive association between glyphosate exposure and breast cancer risk, others have found no association.

Further research is needed to determine the potential health effects of glyphosate expo-
sure on breast cancer risk.

CHAPTER IV

MATERIALS AND METHODS

A. Glyphosate Treatment

Cells were treated with Glyphosate (CAS 1071-83-6, Santa Cruz) at two different periods: during passage 1 (on days 3, 5, and 7), referred to as short-term treatment, and during passages 1 and 2 (on days 3, 5, 7, 13, 15, and 17), referred to as long-term treatment (Figure 1A).

1. Long-Term Treatment:

Long-term glyphosate treatment consisted of a continuous 10^{-5} or 10^{-11} M treatment for 21 days, replenished with every change of media, after which the cells were collected and assayed.

The timetable of glyphosate treatments was based on the published paper of our collaborators (Duforestel et al., 2019). SCp2 and S1 cells were treated with 10^{-5} M and 10^{-11} M glyphosate according to the timetable shown along each experiment, collected and analyzed on day 21 of culture. Explanations for color-coded days are located in the corresponding legend underneath the timeline. For SCp2 cells, glyphosate was diluted in media containing 1% FBS, 1% P/S, and 0.1% insulin (5 µg/mL).

2. Short-Term Treatment:

Short-term glyphosate treatment consisted of a continuous 10^{-5} or 10^{-11} M treatment for 11 days, replenished with every change of media, after which the cells were collected and assayed. SCp2 and S1 cells were treated with 10^{-5} M and 10^{-11} M glyphosate

according to the timetable shown along each experiment, collected and analyzed on day 11 of culture. Explanations for color-coded days are located in the corresponding legend underneath the timeline.

B. Cell Culture

1. HMT-3522 S1 Cells:

The non-tumorigenic ER- negative HMT-3522 S1 human mammary epithelial cells (Briand et al., 1987), with passages ranging from 60 to 72, were cultivated in a mono-layer on plastic (2D culture) in serum-free H14 medium DMEM:F12 medium (GIBCO BRL, St. Louis, MO), containing 250 ng/mL insulin (Boehringer Mannheim, Indianapolis, IN), 10 µg/mL transferrin (Sigma, St Louis, MO), 2.6 ng/mL sodium selenite (BD Biosciences), 10^{-10} M estradiol (Sigma), 1.4 µM hydrocortisone (BD Biosciences), 5 µg/mL Biological prolactin from Ovine (NIDDK-oPRL-21 (AFP-10692C), National Hormone & Peptide Program), and 10 ng/mL epidermal growth factor (EGF; BD Biosciences) (Blaschke, Howlett, Desprez, Petersen, & Bissell, 1994; Plachot & Lelièvre, 2004) at a temperature of 37°C and with 5% CO_2 in a humidified incubator. The H14 medium was replaced every 2-3 days. For 2D cultures, cells were plated on plastic substrata at a density of 2.3x104 cells/cm^2.

The drip method of 3D culture was used to induce the formation of acini. Briefly, cells were plated on Matrigel™ (50 µL/cm^2; Corning, 354234) at a density of 4.2×10^4 cells/cm^2 in the presence of culture medium containing 10% Matrigel™ (Plachot & Lelièvre, 2004; Vidi et al., 2012). To achieve full acinar differentiation (typically achieved on day 8 or 9), EGF was removed from the culture medium from day 7, as stated by Plachot & Lelièvre (2004). In certain experiments, Matrigel™ was mixed with

DMEM:F/12 (Lonza, BE12-719F) at dilutions of 1:5 for S1 cells or 1:20 for SCp2 cells, and then incubated at 37 °C for 4 hours to allow solidification.

2. SCp2 Cells:

For the nontumorigenic ER-positive mouse mammary epithelial cell (SCp2) culture, low passage number (18 to 25) were used throughout. Cells were maintained in DMEM/F12 growth medium containing 1% P/S, 5% FBS (Sigma, St. Louis), and 0.1% insulin (5 µg/mL) at 37°C in a humidified incubator (95% air; 5% CO_2). When reaching 80% confluency, cells were washed with 1x phosphate-buffered saline (PBS) then incubated with 10x trypsin (containing 25.0g porcine trypsin, 10.0g EDTA, 20NA per liter of 0.9% NaCl; Sigma, St. Louis) at 37°C for 1 minute. The cells were washed with 5 mL complete media, and centrifuged at 1000 g for 10 minutes, and the pellet was resuspended in the appropriate amount of media and transferred into new culture plates for maintenance or used for other purposes.

C. Trypan Blue Exclusion Assay

Non-treated S1 cells were plated in 12-well tissue culture plates (2D). The medium was removed, and the cells were subsequently trypsinized and collected. Cells were then diluted in trypan blue at 1:1 ratio (vol/vol) and counted using a hemocytometer. The cells were counted from triplicates on days 4, 6, 8, 14, 16 and 18, following each treatment with glyphosate.

Non-treated SCp2 cells were plated in 6-well plates at a density of 50 x 10^3 cell/mL in their respective culture media. 48 hrs post-plating, the medium was replaced with

DMEM/F12 containing 1% FBS, 1% P/S, 0.1% insulin, and 10^{-5} M or 10^{-11} M glyphosate. First, media was removed, and the cells subsequently trypsinized and collected. Cells were diluted in Trypan Blue (1:1) ratio (V:V) and counted using a hematocytometer. The cells were counted from triplicates on days 2, 5, 7 and 9 following each treatment with glyphosate.

Experiments were repeated at least three times.

D. Transwell Cell Invasion Assay

Six-well culture plates were fitted with inserts (8 μm pore size). The inserts were coated with 400 μL per insert of 1:5 diluted Matrigel™ for S1 cells and 1:20 for SCp2 cells and incubated at 37 °C for 4 hours.

DMEM:F/12 supplemented with 10% FBS was added below the insert. 3 x 10^5 S1 cells were plated in the inserts in DMEM:F/12 supplemented with 1% fetal bovine serum (FBS; Sigma, F-9665). Cells were incubated for 72 hours and were then fixed using 4% formaldehyde in 1x PBS for 20 minutes at room temperature.

8 x 10^4 S1 cells were plated in the inserts in DMEM:F/12 supplemented with 1% FBS. DMEM:F/12 supplemented with 10% FBS was added below the insert. Cells were incubated for 24 hours and were then fixed using 4% formaldehyde in 1x PBS for 20 minutes at room temperature.

The cells towards the inside of the insert were removed using a cotton swab, and nuclei of invading cells at the bottom of the insert were stained with 1 μg/mL Hoechst 33342 (Molecular Probes, H3570) in 1x PBS for 10 minutes at room temperature. The insert was then cut, mounted on a microscope slide in ProLong® Gold antifade reagent

(Invitrogen Molecular Probes, P36930), allowed to dry overnight and sealed. The inserts were examined with a fluorescence microscope, and the number of invading cells was counted and reported as fold change.

E. Immunofluorescence

S1 cells were plated on 4-well chamber slides (3D) (FALCON, 354104) and were stained by immunofluorescence on day 11 as described earlier (Plachot & Lelièvre, 2004). Briefly, cells were washed with 1x PBS and permeabilized with 0.5% peroxide and carbonyl-free Triton X-100 (amersco, LLC 97062208) in cytoskeleton buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES, pH 6.8, 5 mM $MgCl_2$, 1 mM pefabloc, 10 μg/mL aprotinin, 250 μM NaF). Cells were washed twice with cytoskeleton buffer and fixed in 4% formaldehyde, after which cells were washed thrice with 50 mM glycine in 1x PBS and blocked with IF 1x containing 10% Goat Serum for 3 hours at room temperature. Primary antibody used was mouse monoclonal β-catenin (1:50; Santa Cruz Biotechnology, sc-7963). Secondary antibodies conjugated with Alexa Fluor 594 (red) (Invitrogen Molecular Probes, Eugene, OR) were used at the manufacturer's proposed dilutions (1:1000). Nuclei were counterstained with 1 μg/mL Hoechst, and cells were mounted in ProLong® Gold antifade reagent, allowed to dry overnight and sealed. The slides were then examined and imaged with a laser scanning confocal microscope (Zeiss, LSM710). A minimum of 50 acini were analyzed per group.

F. Preparation of Whole Cell Protein Extracts and Western Blot Analysis

S1 cells were plated in T-75 tissue culture flasks (2D). Cells were washed with 1x PBS and harvested from 2D cultures by scraping with lysis buffer (50 mM Tris-HCl,

150 mM NaCl, 1% Nonidet P-40, 0.5% Sodium Deoxycholate, and 4% Protease Inhibitors) and were collected by centrifugation at 15 000 g. Cells were lysed on day 21 for long-term and day 11 for short-term. Proteins were quantified using the DC protein assay (Bio-Rad, 5000116). For Western blot analysis, equal amounts of proteins (50 μL) were separated on 12% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes that were subsequently blocked with 5% low-fat milk in tris-buffered saline-Tween 20 (TBST) and immunoblotted with the following: mouse monoclonal antibodies against Cx43 (1-2 μg/mL; Santa Cruz Biotechnology, sc- 271837, and 5 μg/mL; Invitrogen, 13-8300), and β-catenin (2 μg/mL; Santa Cruz Biotechnology, sc-57535). Secondary antibodies conjugated to goat anti-mouse (Abcam, ab6787), were used at 0.13 μg/mL. Equal protein loading was verified by immunoblotting for GAPDH (mouse monoclonal, 0.4-1 μg/mL; Santa Cruz Biotechnology, sc-47724). Protein levels were quantified using ImageJ software and normalized to GAPDH.

G. Total RNA Isolation and Quality Control

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, cat# 74134) for total RNA isolation from SCp2 cells according to the manufacturer's instructions. Purity and concentration of RNA samples were examined spectrophotometrically by absorbance measurements at 260, 280 and 230 nm using the NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). A260/A280 ratios between 1.8 and 2.1 were considered acceptable.

H. Quantitative Real-Time PCR (qRT-PCR)

Reverse transcription of 10 ng of the total RNA was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions and as previously described by Nassar et al. (2017). Briefly, small nuclear RNA RNU6B, miR-182-5p primers and probes were purchased as part of the TaqMan microRNA Assays Kit (Applied Biosystems, USA) with validated efficiency. cDNA synthesis was carried out for miR-183-5p in each reaction with the endogenous control, RNU6B. RT-qPCR was performed using BioRad CFX96 Real Time System, C1000 Thermal Cycler (Germany). Reactions using 10 µL of SYBR Green JumpStart Taq ReadyMix (SIGMA S4438), 0.4 µL of the corresponding microRNA primer set from Hairpin-it^{TM} miRNA and U6 snRNA normalization RT- PCR quantification kit (GenePharma), 5.6 µL of DEPC treated water, and 4 µL of cDNA were performed in duplicates for each miRNA probe. cDNA Synthesis and RT-qPCR were repeated twice for each sample and each plate included no reverse transcription control (NRT), no template control (NTC). The cycling conditions were 94 °C for 3 minutes and 40 cycles of 94 °C for 15 seconds and an annealing temperature of 55 °C for 25 seconds, and 12°C for 25 seconds. The relative expression of miRNA was determined using the ΔCt equation.

I. Reverse-Transcription PCR

1 µg of total RNA was reverse transcribed to cDNA using Quantitect Reverse Transcription Kit (QIAGEN, Cat No./ID: 205311) according to the manufacturer's protocol. RT-PCR was performed using SYBR Green JumpStart Taq ReadyMix (SIGMA, S4438) at 95 C for 2 minutes (Step 1), 95 for 15 seconds (Step 2), 56 for 13 seconds

(Step 3), 72 for 1 minute (Step 4), 72 for 10 minutes (Step 5). Steps 1-4 were repeated 39 times. Products were amplified using primers for the below genes.

- Mus Musculus Beta Casein Transcript mRNA sequence

Fwd: 5’ GTGGCCCTTGCTCTTGCAAG 3’

Rev: 5’ AGTCTGAGGAAAAGCCTGAAC 3’

- GAPDH Glyceraldehyde 3 phosphate dehydrogenase mus musculus mRNA sequence

Fwd:5’CATGGCCTTCCGTGTTCCTA 3’

Rev: 5’ CCTGCTTCACCACCTTCTTGAT 3’

To quantify changes in gene expression, fold changes were normalized to GAPDH.

J. Image Processing

Images of immunofluorescence labeling were recorded using LSCM fluorescent confocal microscope (LSM 410, Zeiss, Germany). Images were processed using ZEN lite software and ImageJ (http://imagej.nih.gov/ij/) and assembled using Adobe Photoshop® 6.0 (Adobe Systems, San Jose, CA).

K. Statistical Analysis

Data were presented as means ± standard error of the mean (SEM) and statistical comparisons were done using Microsoft Excel and GraphPad Prism 3.0 software (GraphPad Software Inc, San Diego, CA). Non-paired and paired t-test was used for comparison of two groups whereas one-way ANOVA was employed for three or more groups of treatments. Significance levels was at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

L. Zymography Assay (Substrate-Gel Electrophoresis) for Gelatinase Activities

Culture media were collected from the respective SCp2 cell cultures after 21 days of glyphosate exposure and stored at -80°C. Gelatinase activity in the collected media was analyzed using the method described by (Talhouk et al., 2008). Briefly, equal sample volumes mixed in 1:1 ratio (V:V) with 2x sample buffer were loaded and run on 7% polyacrylamide gels impregnated with gelatin (4.5 mg/mL). The gels were run in 1x electrophoresis running buffer (0.0025 M TrisHCL, pH 8.3, 0.192 M glycine, 0.1% SDS). After electrophoresis, gels were washed once for one hour with wash buffer (substrate buffer with 2.5% Triton X-100) at room temperature and then incubated for 24 hrs in substrate buffer (50 mMTris-HCL, 5 mM CaCl2, 0.02%NaN3, pH 8.0) at 37°C. The gels were stained for 2 hrs at room temperature in 0.05% Comassie blue R- 250 (Sigma, St. Louis, Missouri, USA), in 50% methanol and 10% acetic acid and distained in distilled deionized water for 24 hrs. The gelatinases appeared as clear white bands on darkly stained blue gels then colors were inverted using ImageJ software in order to visualize the gelatinases as black bands against a white background as presented. Peak areas of MMP-9 bands were quantified using ImageJ in triplicates and data is represented as the average fold increase of MMP-9 band peak area (Arbitrary Basal Density) of three experiments ± SEM (AU ± SEM).

M. Ingenuity Pathway Analysis (IPA)

Chemicals, genes, and miRNAs of interest were added into the IPA pathway interface (Ingenuity Systems, Redwood City, CA, USA) for analysis. Using IPA, the molecules were assigned into networks and biological functions on the basis of right-tailed Fisher's test ($P<0.05$).

CHAPTER V

RESULTS

Our study involved the application of ductal non-tumorigenic HMT-3522 S1 human mammary epithelial cells cultured in 3D conditions to generate acini that are well-differentiated, growth-arrested, and basally polarized. Additionally, we used lobular mouse mammary epithelial SCp2 cells, which can be differentiated through the addition of lactogenic hormones and appropriate basement membrane components, resulting in the exclusive expression of β-casein as a differentiation marker.

Cells were treated either during passage 1 (days 3, 5, and 7), referred to as short-term treatment, or during passages 1 and 2 (days 3, 5, 7, 13, 15, and 17), referred to as long-term treatment.

Data was collected during culture at days indicated after treatment, per respective assays, as described in Materials and Methods section.

A. 10^{-5} M glyphosate treatment decrease non-tumorigenic human mammary epithelial S1 cell counts after the 3rd treatment (short-term) and the 6th treatment (long-term).

Viable S1 cell count upon glyphosate treatment was assessed by trypan blue staining. The average number of S1 cells treated with 10^{-11} M glyphosate increased similarly to control untreated cells during passages 1 and 2 (Fig. 1B). Although treatment with 10^{-5} M of glyphosate exhibited similar trends to those of 10^{-11} M on days 4 and 6 during passage 1, and 14 and 16 during passage 2, there was a significant dip in the number of S1 cells on days 8 during passage 1 (8 days after plating) and 18 during passage 2 (8 days after plating) to ~ 10×10^4 and 9×10^4 cells, respectively, compared to the control untreated cells (~17×10^4 - 19×10^4 cells) (p value <0.001). The number of dead cells

was negligible and did not differ between the different treatments and controls at the different time points.

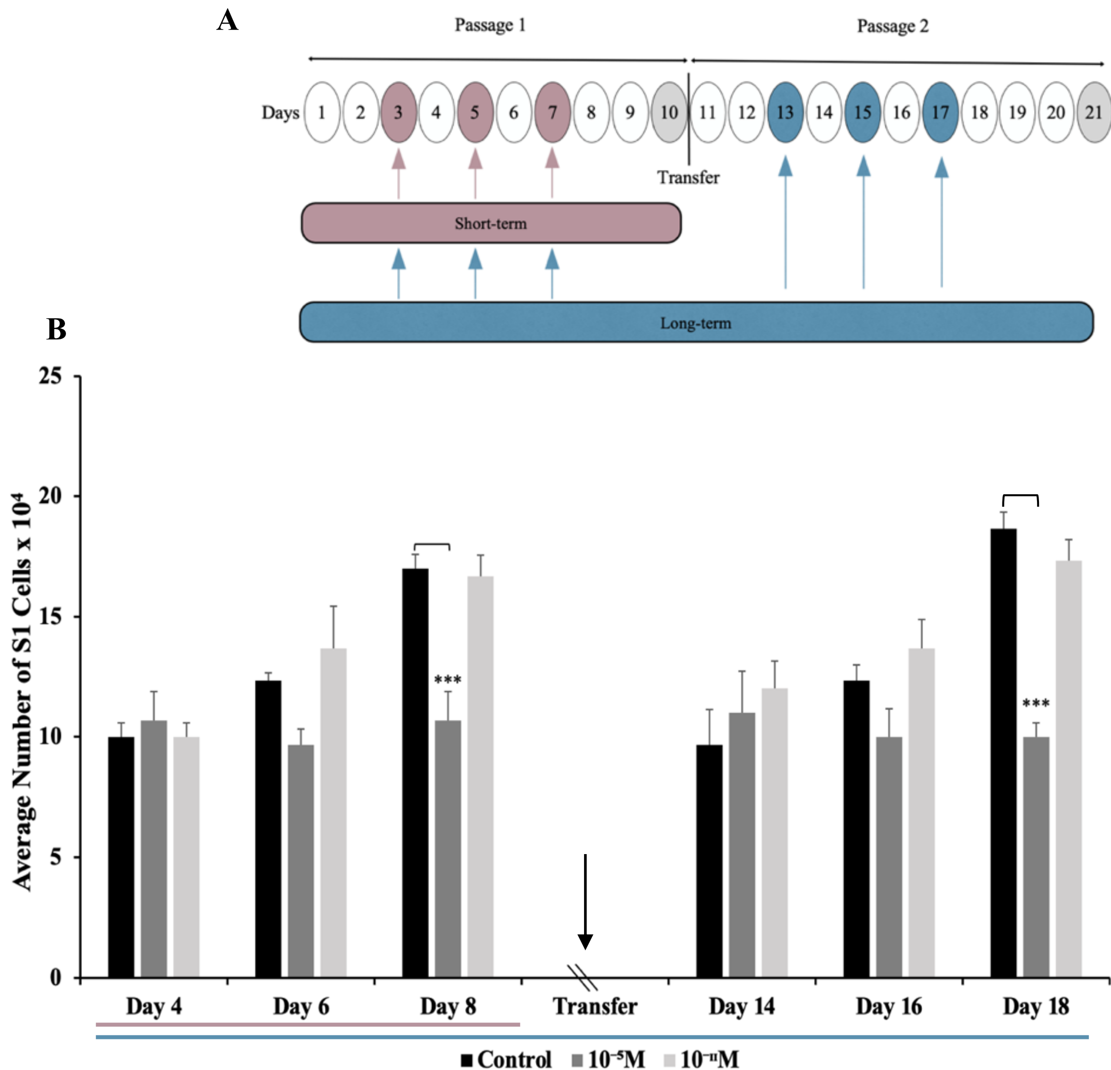

Figure 1: Non-tumorigenic human mammary epithelial S1 cell counts decrease on day 8 in short-term treated cells and on day 18 (or day 8 post-transfer) in long-term treated cells with 10^{-5} M, but not with 10^{-11} M glyphosate. (A) Treatment plant for S1 cells cultured under 2D conditions in 12-well plates and treated with 10^{-5} M glyphosate for short-term (covering 1 passage) or long-term (covering 2 passages) **(B)** Bar graph showing a significant decrease in S1 cell counts 8 days or 3 treatments after plating for passages 1 and 2. No differences in the growth profile of 10^{-11} M glyphosate-treated cells were noted. The values depicted are the mean ±SEM from three separate experiments. *** denotes a p value < 0.001 compared to untreated controls for each treatment using t-test.

B. Glyphosate treatment enhances invasion in nontumorigenic human breast epithelial S1 cells across reconstituted basement membrane.

To determine the effect of short-term and long-term treatment with 10^{-5} M and 10^{-11} M glyphosate on S1 cell invasion ability through Matrigel compared to the non-treated control, cells were counted 72 hours post-transfer into trans-well inserts. Short-term 10^{-5} M and 10^{-11} M glyphosate treatment of S1 cells showed a significant increase of 1.5-fold and 1.6-fold in the number of Matrigel-invading cells, respectively, compared to control untreated cells (p value <0.05) (Fig. 2A). Additionally, long-term 10^{-5} M and 10^{-11} M glyphosate treatment of S1 cells showed a significant increase of ~1.3-fold and 1.2-fold, respectively, compared to control untreated cells (p value <0.05) (Fig. 2B).

A

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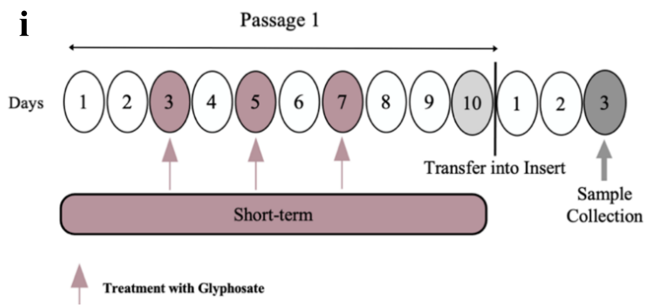

A

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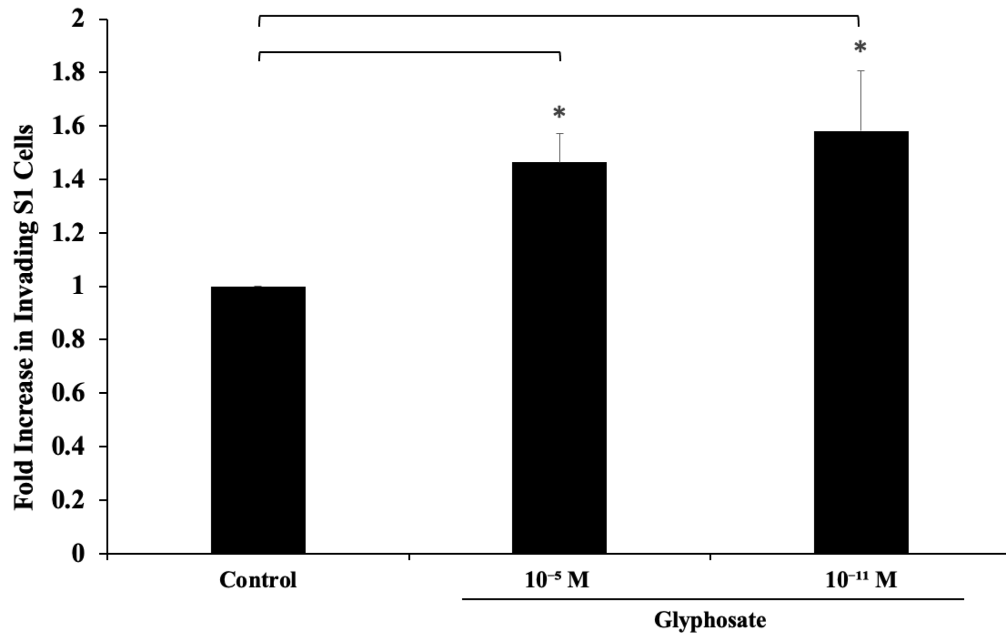

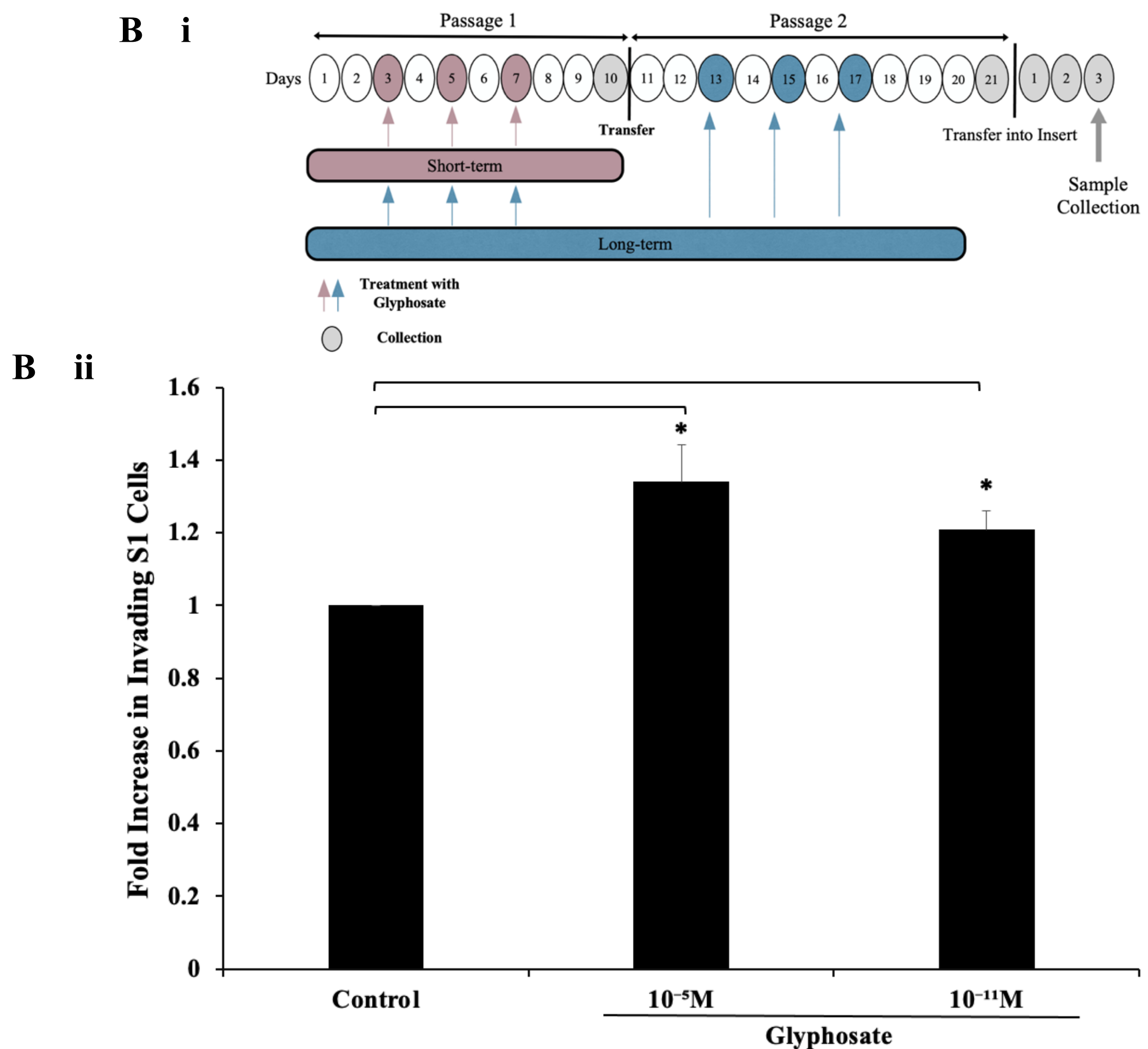

Figure 2: Short-term and long-term glyphosate treatment enhances invasion in non-tumorigenic human mammary epithelial S1 cells. (A-i) S1 cells cultured in diluted Matrigel (1:5) (Fostok *et al*., 2019) and subjected to short-term 10^{-5} M or 10^{-11} M glyphosate treatment according to the treatment plan **(A-ii)** showed a significant fold increase in the number of invading cells compared to untreated controls. **(B-i)** S1 cells cultured in diluted Matrigel (1:5) and subjected to long-term 10^{-5} M or 10^{-11} M glyphosate treatment according to the treatment plan **(B-ii)** showed a significant fold increase in number of Matrigel-invading cells compared to untreated controls. Invasion was assessed by trans-well invasion assay; invading S1 cells were stained by Hoechst 72h post-transfer into inserts, followed by counting. The values depicted are the mean ±SEM from three separate experiments. $*$ denotes a p value < 0.05 compared to the untreated control using t-test.

C. Short-term glyphosate treatment of S1 3D cultures alters acinar structure when added pre-lumen assembly or both pre- and post-lumen assembly, but not post-lumen assembly.

The non-tumorigenic human mammary epithelial HMT-3522 S1 cell line grows into 3D differentiated acinar-like spheroids by day 7 on Matrigel, which are around 30 μm in diameter and have a single layer of cells encircling a central lumen with apicolateral polarity (Peterson et al., 1992; Vidi et al., 2013). To determine whether short-term glyphosate treatment could affect the organization and differentiation of the mammary epithelium through different stages of acinar formation, S1 cells on Matrigel were subjected to short-term glyphosate treatment pre-lumen, post-lumen, as well as pre- and post-lumen assembly, after which acinar 3D morphogenesis and lumen formation were assessed. For treatments performed pre-lumen assembly (Fig. 4C), 68% of the control untreated S1 acini displayed typical lumen structures enclosed within a single layer of cells, but only 51% and 47% of the S1 acini treated for short periods with 10^{-5} M and 10^{-11} M glyphosate, respectively, had normal morphology with undisrupted lumen (p value<0.05). Additionally, treatments done pre- and post-lumen assembly showed significant decrease in the percentage of acini with a normal lumen with 10^{-5} and 10^{-11} M glyphosate (~52%, p value<0.05) when compared to the control untreated group (66%) (Fig. 4D). Conversely, S1 cells treated with 10^{-5} M and 10^{-11} M glyphosate post-lumen assembly (Fig. 4E) showed no significant change in the percentage of normal acini (61% and 57%, respectively) when compared to the control untreated group (62%).

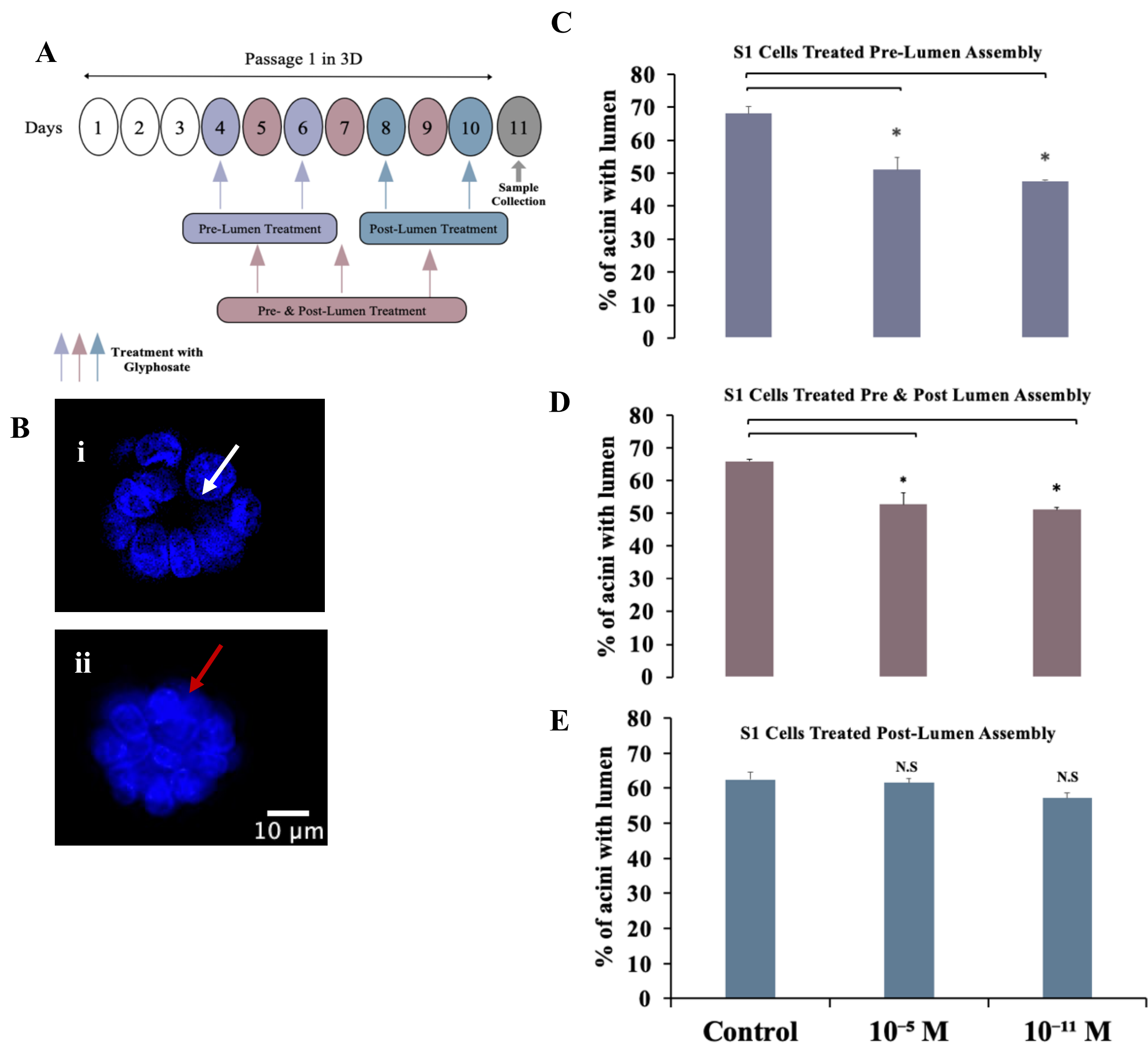

Figure 3: Lumen formation in 3D cultures of mammary epithelial S1 cells is disrupted upon short-term glyphosate treatment pre-lumen assembly and both pre- and post-lumen assembly, but not upon treatment post-lumen assembly. (A) Treatment plan for S1 cells cultured under 3D conditions in 4-well cell culture chambers and treated with 10^{-5} M and 10^{-11} M glyphosate for short-term. Acini of **(B-i)** control untreated S1 cells (0 *M*) and **(B-ii)** S1 cells subjected to short-term glyphosate treatment at 10^{-5} or 10^{-11} M were stained with Hoechst (blue) and scored for lumen formation. White arrows point at acini with normal monolayered lumen, while red arrows point at those with disrupted multi-layered lumen. **(C)** Bar graph showing a decrease in the percentage of acini with proper lumen assembly from 69% to ~50% in short-term glyphosate-treated (10^{-5} and 10^{-11} M) S1 cells pre-lumen assembly. **(D)** Bar graph showing a significant difference in the percentage of acini with proper lumen assembly between control untreated (0 *M*) and short-term glyphosate-treated (10^{-5} and 10^{-11} M) S1 cells pre- and post- lumen assembly from ~65% to ~50%. **(E)** Bar graph showing no significant difference in the percentage of acini with proper lumen assembly in control untreated (0 *M*) and short-term glyphosate-treated (10^{-5} and 10^{-11} M) S1 cells post-lumen assembly. Cells were fixed on day 11 and stained using Hoechst. One hundred acini were scored for each condition in every replicate. Each bar represents triplicate analyses of mean ± SD. $* \ p < 0.05$ compared to the untreated control using t-test analysis.

D. Long-term glyphosate treatment disrupts lumen formation ability in 3D cultures of human breast epithelial S1 cells.

To determine whether long-term glyphosate treatment could influence the organization of the breast epithelium, S1 cells were subjected to long-term treatment pre- and post-lumen assembly only, after which acinar morphogenesis and lumen formation were assessed. While 61% of the control untreated S1 acini displayed typical lumen structures enclosed within a single layer of cells (Fig. 4B-i control and 4C), only 34–35% of the S1 acini treated for long periods with 10^{-5} and 10^{-11} M glyphosate, respectively, had normal morphology with undisrupted lumen.

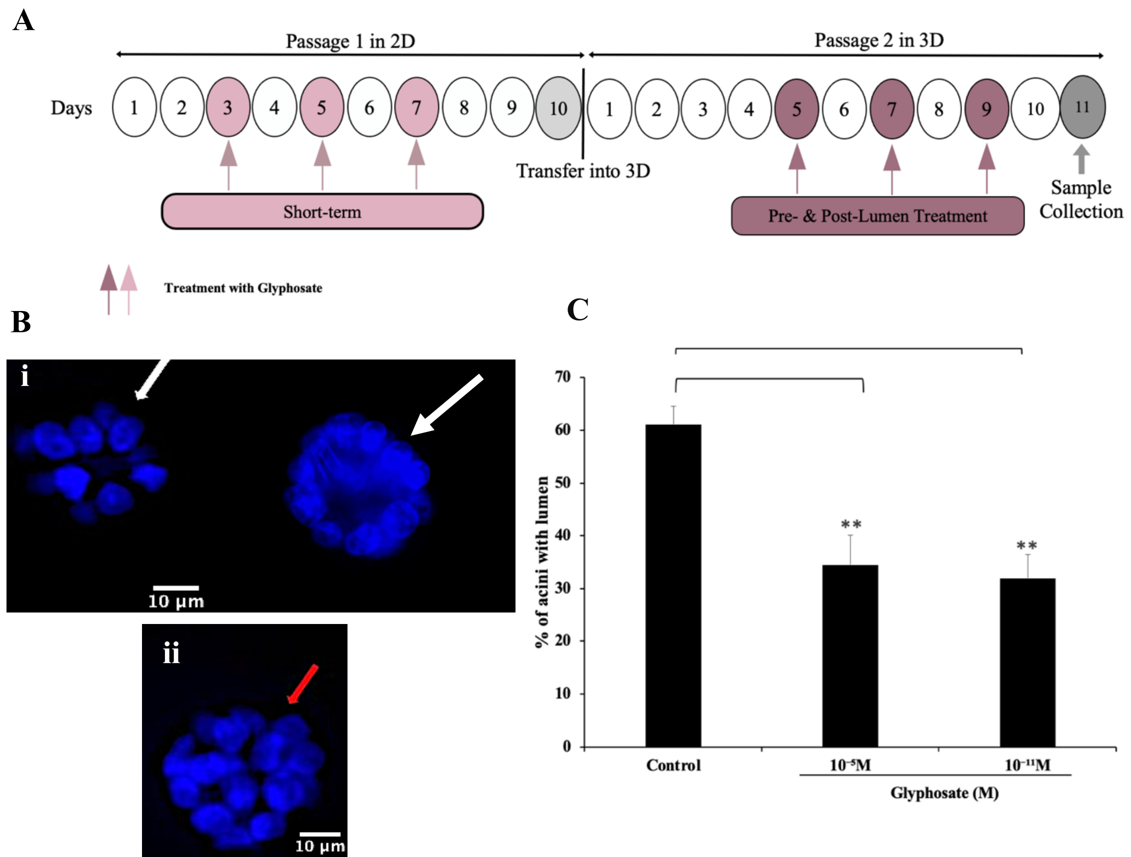

Figure 4: Long-term glyphosate treatment disrupts lumen formation in 3D cultures of human breast epithelial S1 cells. (A) Treatment plan for S1 cells cultured under 2D conditions then transferred into 3D 4-well cell culture chambers and treated with 10^{-5} M and 10^{-11} M glyphosate for long-term. Acini of **(B-i)** control untreated S1 cells (0 *M*) and **(B-ii)** S1 cells subjected to long-term glyphosate treatment at 10^{-5} and 10^{-11} M were stained with Hoechst (blue) and scored for lumen formation. White arrows point at acini with normal monolayered lumen, while red arrows point at those with disrupted lumen. **(C)** Bar graph showing more significant decrease in the percentages of acini with undisrupted monolayered lumen in S1 cells subjected to long-term glyphosate treatment compared to short-term treated ones, from 60% to ~34-35%. One hundred acini were scored for each condition in every replicate. Each bar represents triplicate analyses of mean ± SD. ** $p < 0.01$ compared to the untreated control using t-test.

E. Long-term glyphosate treatment does not affect expression levels of polarity markers β-catenin and Cx43 in 10^{-5} and 10^{-11} M glyphosate-treated 2D extracts of S1 cells.

We have previously shown that S1 cell's typical acinar morphology and epithelial cell polarity are both affected by Cx43 expression (Bazzoun et al., 2019). Moreover, Cx43 assembly into GJs and its association with ZO-2 and β-catenins is shown to be essential

to mammary epithelial differentiation (Talhouk et al., 2008). To determine whether glyphosate affects expression levels of Cx43 and *β*-catenin, a Cx43-associated polarity protein, western blot analyses were performed. No significant changes were observed in Cx43 and *β*-catenin expression levels upon treatments with both concentrations of glyphosate when compared to the untreated control (Fig. 5A). Further quantitative analysis of the bands obtained through western blotting confirmed the non-significant change in normalized *β*-catenin (Fig. 5B) and Cx-43 (Fig. 5C) expression levels upon 10^{-5} and 10^{-11} M glyphosate treatment.

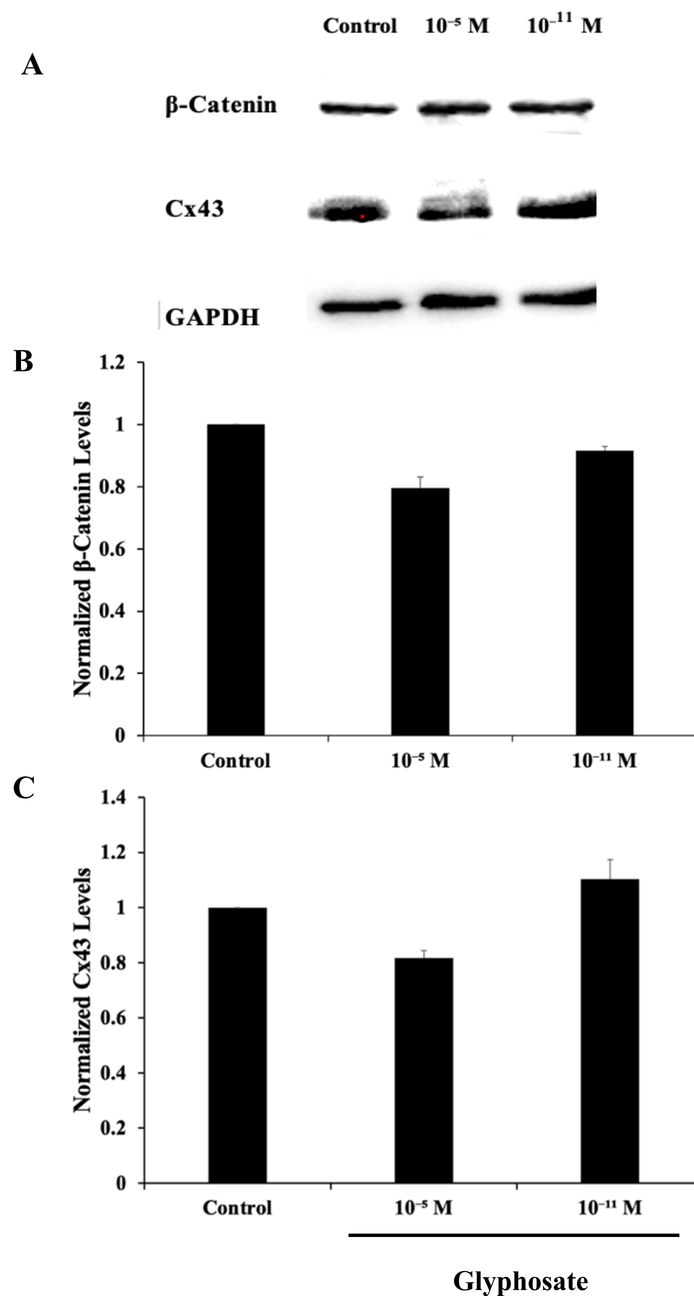

Figure 5: Western blot analysis show no significant change in β-catenin and Cx43 levels of long-term glyphosate treated S1 cells. (A) Representative western blot comparing the expression of *β*-catenin and Cx43 in 10^{-5} and 10^{-11} M glyphosate-treated S1 cells and the untreated control. GAPDH was used for normalization of protein loading. **(B)** Quantification of Cx43 and **(C)** *β*-catenin levels normalized to GAPDH showing no significant change compared to the untreated control. The analysis depicted are the means ± standard error of the mean (SEM) from two independent experiments.

F. Short-term and long-term glyphosate treatment alters β-catenin localization in 3D cultures of human breast epithelial S1 cells.

Data from our laboratory has shown that S1 acini with correct morphology display apicolateral distribution of *β*-catenin and gap junctional complexes (Bazzoun et al., 2019). To determine whether *β*-catenin cellular distribution differ upon glyphosate treatment, immunofluorescence was used. It was noted that in the untreated control, *β*-catenin is localized apicolaterally (Fig. 6B Control). However, the redistribution was noted in short-term treated cells whereby *β*-catenin was redistributed across the entire cell membrane (basolaterally). Furthermore, scoring of acini with apicolateral distribution of *β*-catenin showed and confirmed a significant decrease from ~70% in the untreated control group, to 57% and 39% upon 10^{-5} M (p value<0.01) and 10^{-11} M (p value<0.001) glyphosate treatment, respectively (Fig. 6C).

To further ascertain whether *β*-catenin cellular distribution maintains a basolateral distribution pattern upon long-term glyphosate treatment, immunofluorescence was used on long-term treated S1 cells. It was indeed noted that *β*-catenin displayed a diffused localization in treated cells compared to its apicolateral localization of untreated controls (Fig. 7B). This was further validated upon scoring of acini with apical *β*-catenin, where the percentage decreased from ~73% to 42% and 40% upon 10^{-5} and 10^{-11} M glyphosate, respectively (p value<0.001) (Fig. 7C).

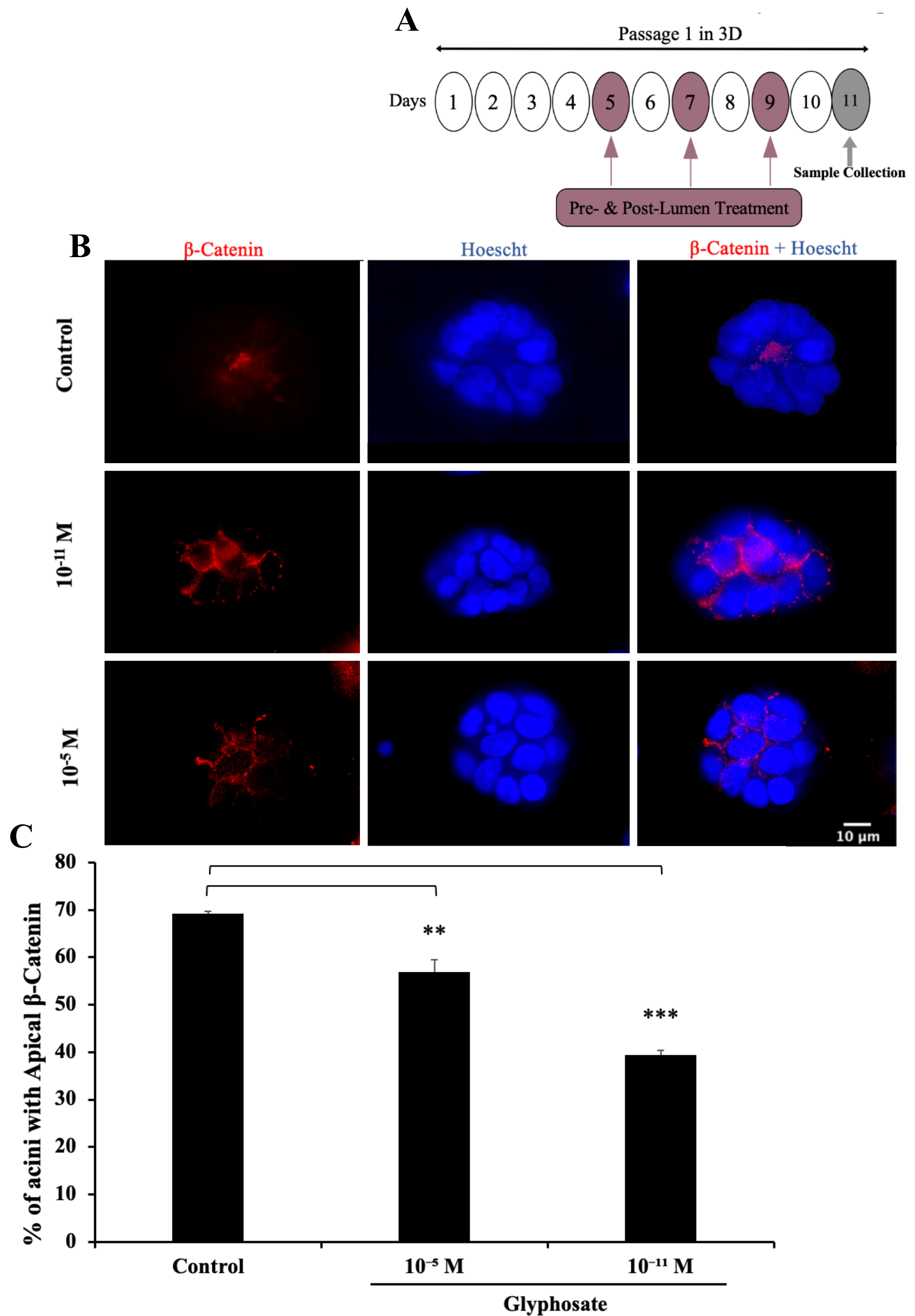

Figure 6: 10^{-5} and 10^{-11} M short-term glyphosate treatment alters β-catenin localization in 3D cultures of human breast epithelial S1 cells. (A) Treatment plan for S1 cells cultured in 3D and treated for short-term with 10^{-5} M and 10^{-11} M glyphosate. **(B)** Representative S1 acini immunostained for *β*-catenin (red) and counterstained with Hoechst

(blue). The upper lane shows a control untreated S1 acinus with undisrupted monolayered lumen and apicolateral *β*-catenin localization. The middle lane shows an S1 acinus following short-term glyphosate treatment at 10^{-11} M, and the lower lane shows an S1 acinus following short-term glyphosate treatment at 10^{-5} M. Both 10^{-5} and 10^{-11} M short-term treatment reveal relocalization of *β*-catenin to a basolateral distribution across the entire cell membrane. **(C)** Quantification of *β*-catenin localization shows significant redistribution of *β*-catenin from the apicolateral to the basolateral domain in S1 acini with monolayered lumen after short-term glyphosate treatment at 10^{-5} and 10^{-11} M. Localization of *β*-catenin was evaluated under fluorescent microscopy. One hundred acini from every replicate were visualized, and each acinus was scored for polarity based on the *β*-catenin (red) localization as apicolateral versus basolateral. Each bar represents triplicate analyses of mean ± SD. ** $P < 0.01$ and *** $P < 0.001$ compared to the untreated control using unpaired t-test.

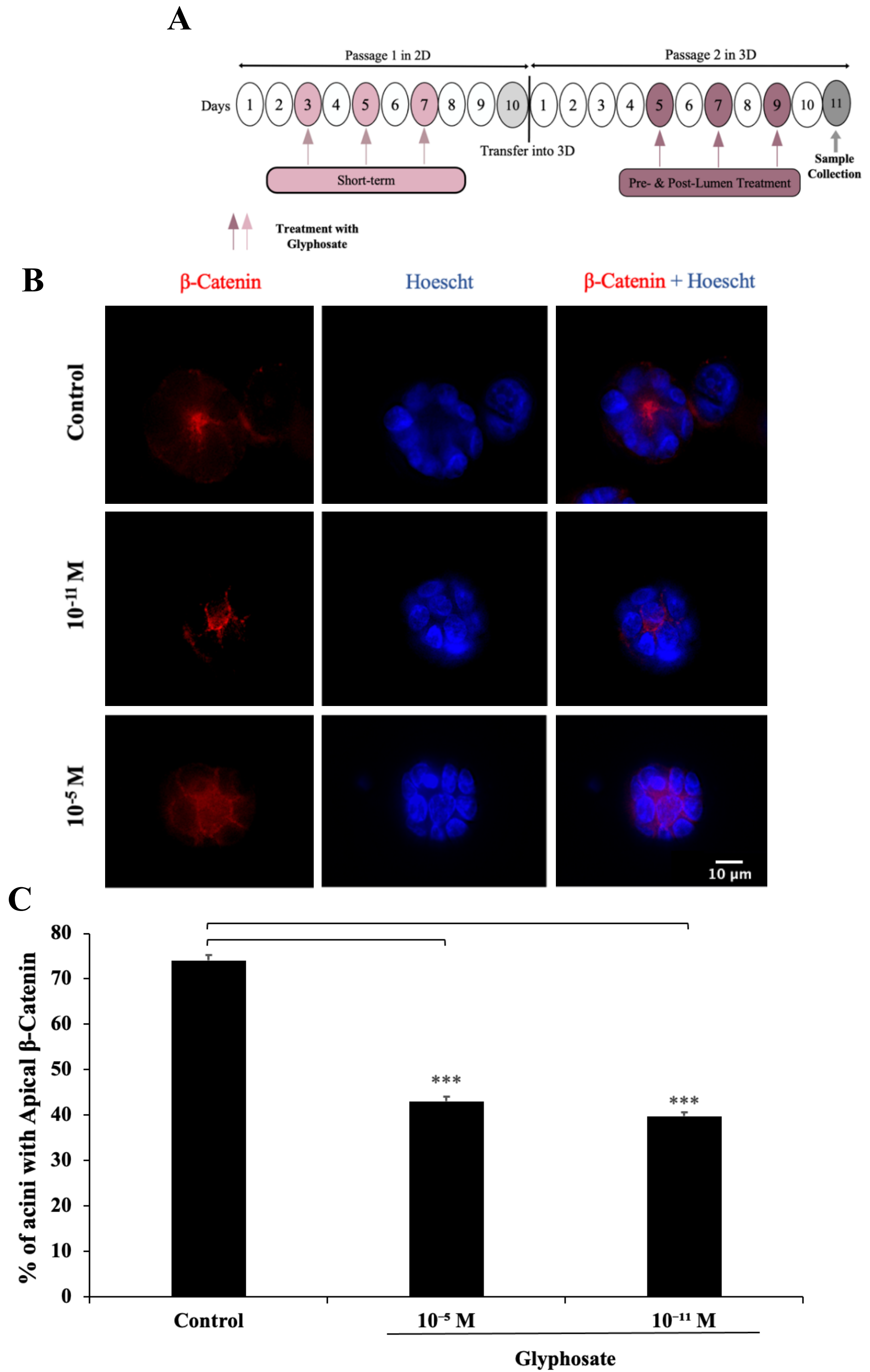

Figure 7: 10^{-5} and 10^{-11} M long-term glyphosate treatment alters β-catenin localization in 3D cultures of human breast epithelial S1 cells. (A) Treatment plan for S1 cells cultured in 2D then transferred to 3D and treated for long-term with 10^{-5} M and

10^{-11} M glyphosate. **(B)** Representative S1 acini immuno-stained for *β*-catenin (red) and counterstained with Hoechst (blue). The upper lane shows a control untreated S1 acinus with undisrupted monolayered lumen and apicolateral *β*-catenin localization. The middle lane shows an S1 acinus following short-term glyphosate treatment at 10^{-11} M, and the lower lane shows an S1 acinus following short-term glyphosate treatment at 10^{-5} M. Both 10^{-11} and 10^{-5} long-term treatments reveal relocalization of *β*-catenin from an apicolateral to a basolateral distribution pattern. **(C)** Quantification of *β*-catenin localization shows significant redistribution of *β*-catenin from the apicolateral to the basolateral domain in S1 acini after long-term glyphosate treatment at 10^{-5} and 10^{-11} M. Localization of *β*-catenin was evaluated under fluorescent microscopy. One hundred acini from every replicate were visualized, and each acinus was scored for polarity based on the *β*-catenin (red) localization as apicolateral versus basolateral. Each bar represents triplicate analyses of mean ± SD. *** $P < 0.001$ compared to the untreated control using unpaired *t*-test.

G. Short-term and long-term glyphosate treatment does not affect growth rate of normal mouse mammary epithelial cells (SCp2).

Next, we sought to investigate the effect of glyphosate on loss of differentiation, marked by the downregulation of ß-casein, and tumor initiation in lobular mouse mammary epithelial SCp2 cells.

To investigate the effect of exposure to 10^{-5} and 10^{-11} M glyphosate on SCp2 cell growth rate compared to the non-treated control, exposed cells were counted at the corresponding time points, days 2, 5, and 8 during passage 1 (short-term treatment), and days 12, 15, and 18 during passage 2 (long-term treatment) (Fig. 8A). No significant difference in the growth rate of SCp2 cells was observed between treatments with both concentrations and control non-treated cells whether for short-term or long-term (Fig. 8B). The number of dead cells was negligible and did not differ between the different treatment and controls at the different time points.

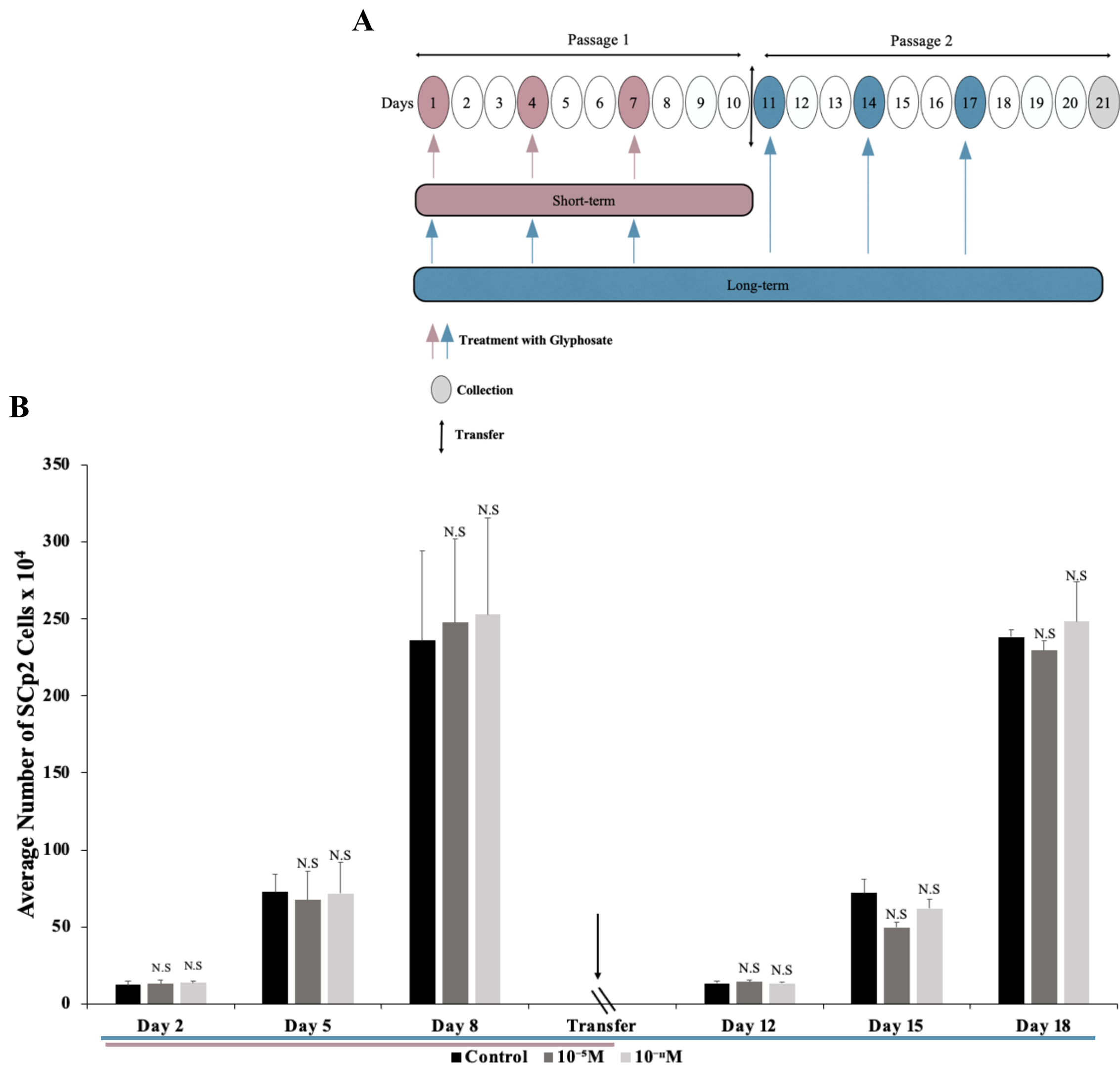

Figure 8: Short-term and long-term glyphosate treatment does not affect growth rate of mammary epithelial SCp2 cells. (A) Glyphosate exposed SCp2 cells for short-term and long-term, were cultured in 2D conditions in 6-well plates. **(B)** Cell count was assessed using trypan blue exclusion assay at day 2, 5, 8, 12, 15, and 18 (following each treatment). Number of cells was not affected by the addition of glyphosate at the different timepoints. The values depicted are the means ± standard error of the mean (SEM) of cell counts x 10^4 from three independent experiments. N.S = Not significant.

H. Long-term 10^{-11} M glyphosate treatment enhances invasion in nontumorigenic mouse mammary epithelial SCp2 cells.

To assess whether glyphosate treatment induces cell invasion through matrigel, trans-well invasion assay was performed by seeding equal numbers of SCp2 cells over trans-well filters (having pores of 8 μm in diameter) coated with 1:20 diluted matrigel.

After 48 hours, the number of invading SCp2 cells was viewed under fluorescent microscope and counted (Fig. 9B). Glyphosate treatment increased the number of invading SCp2 cells by ~8-folds when treated for long-term with 10^{-11} M glyphosate (p value < 0.05) (Fig. 9C), whereas treatment with 10^{-5} M glyphosate exhibited a non-significant ~4-fold increase in invading SCp2 cells.

Additionally, conditioned media were collected and assayed for MMP-9 by zymography. Active MMP-9 levels were upregulated upon treatment with 10^{-5} M and 10^{-11} M of glyphosate compared to the untreated control, as shown in the zymogram (Fig. 9D). 10^{-11} M treatment exhibited higher levels of MMP-9 compared to the 10^{-5} M treated cells, with respect to the control untreated cells.

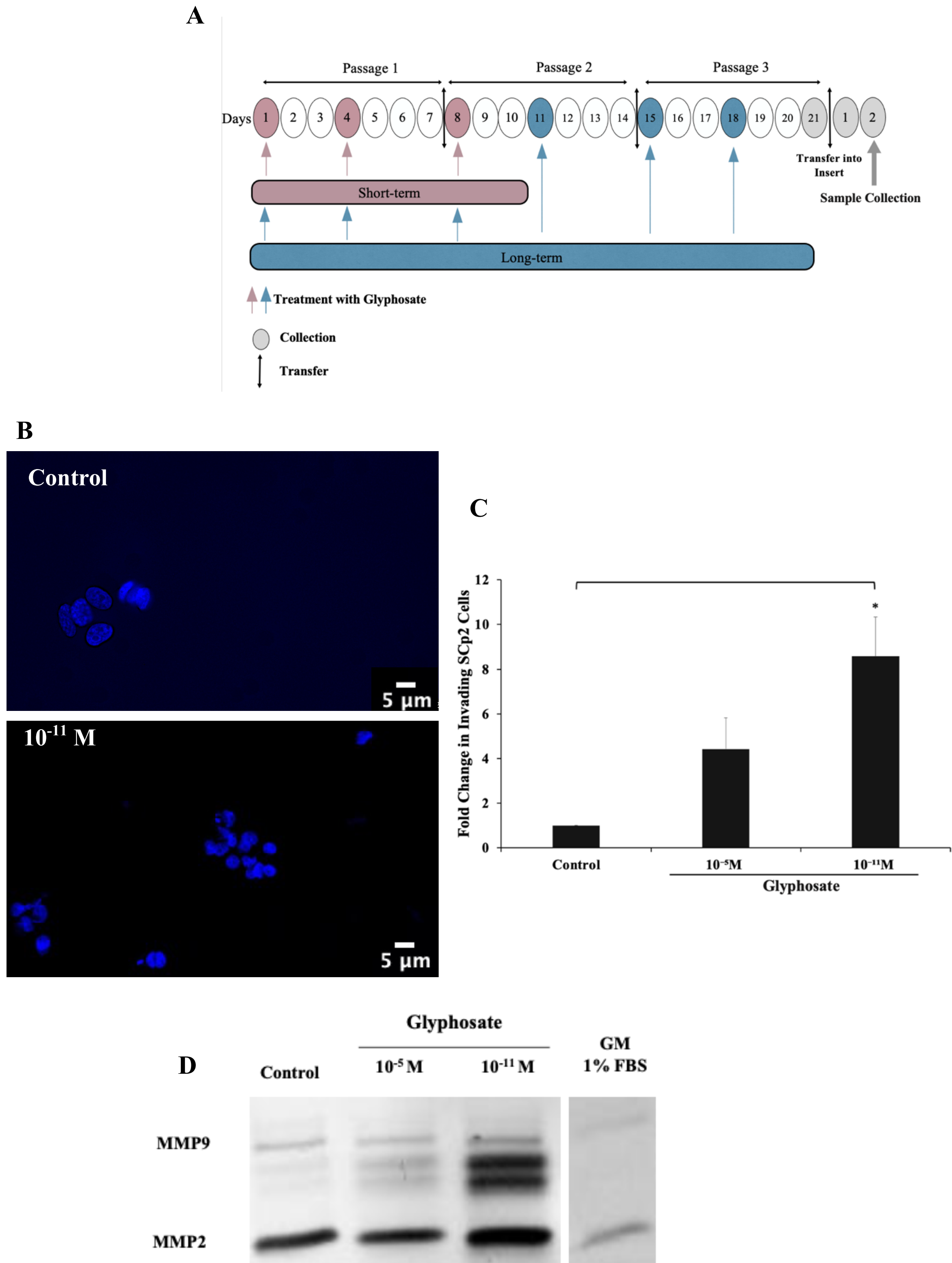

Figure 9: 10^{-11} M long-term glyphosate treatment induces an invasive phenotype in nontumorigenic SCp2 mammary epithelial cells. (A) Treatment plan for SCp2 cells treated for long-term in 2D culture, then transferred to trans-well inserts for 24 hours. **(B)** Representative images of untreated control and 10^{-11} M glyphosate treated SCp2 cells invading across 1:20 diluted Matrigel. Invading SCp2 cells were counted after fixation

and Hoechst staining. **(C)** Histogram showing an increase in the fold change of the number of invading SCp2 cells after treatment with 10^{-11} M glyphosate. The values depicted are the mean ±SEM from three separate experiments, ∗ denotes a p value < 0.05 compared to controls for each treatment using t-test. **(D)** Gelatin zymography of SCp2 conditioned media collected from non-treated control cells and glyphosate treated cells (10^{-5} and 10^{-11} M) upon reaching 80% confluency. GM=Growth Media containing 1% FBS (used as a negative control).

I. 10^{-11} M long-term glyphosate treatment downregulates differentiation marker β-Casein expression in SCp2 cells.

Under differentiation-permissive conditions ensured by the addition of lactogenic hormones, such as prolactin and suitable basement membrane components, SCp2 cells produce β-casein as a differentiation marker. In order to check whether glyphosate treatment affects differentiation of SCp2 cells, PCR was performed on mRNA extracted from SCp2 cells which were induced to differentiate using EHS drip. Samples run on agarose gel showed a decrease in β-casein expression in 10^{-11} M glyphosate-treated SCp2 cells, compared to the untreated positive control (Fig. 10A). Quantification of bands further validated a significant downregulation of β-casein in glyphosate-treated cells (p value<0.05) (Fig. 10B).

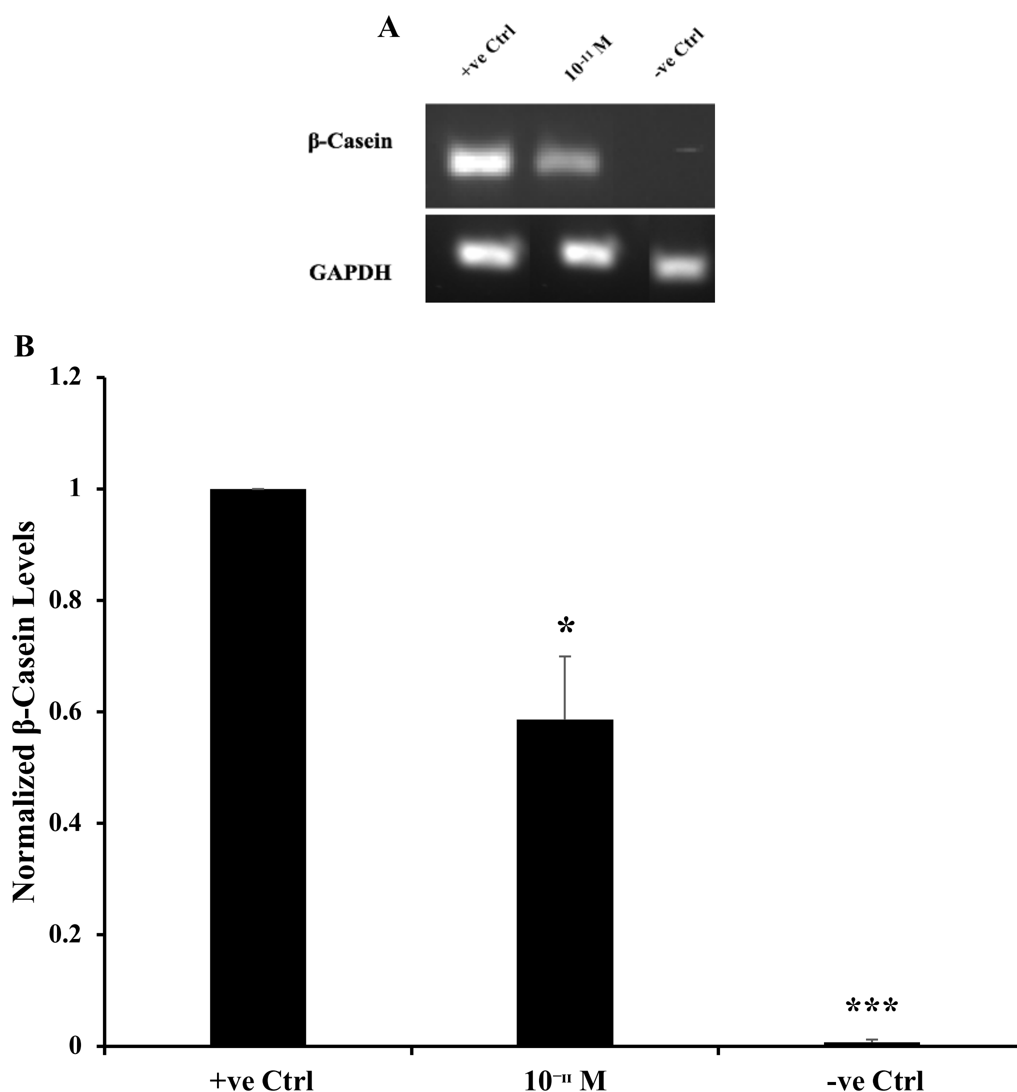

Figure 10: 10^{-11} M long-term glyphosate treatment disrupts β-Casein expression in SCp2 cells on drip. **(A)** A representative agarose gel showing beta-casein mRNA levels after treatment of SCp2 cells with 10^{-11} M of glyphosate on drip. As a negative control for drip, SCp2 cells were seeded on plastic and supplemented with non-differentiation media (NDM) lacking prolactin. **(B)** Quantification of bands shown in the agarose gel using ImageJ show significant decrease in β-Casein levels. β-Casein mRNA levels were normalized with GAPDH expression. Statistical analyses were obtained from two independent experiments; * denotes $p < 0.05$ and *** denotes $p < 0.001$.

J. Long-term glyphosate exposure upregulates miR-183 expression in mammary epithelial SCp2 cells.

To evaluate whether glyphosate influences onco-miR-183-5p in SCp2 cells, levels of miR- 183-5p were assessed by RT-qPCR. The results revealed an estimated 1.5- and 4-fold increase in miR-183-5p expression in SCp2 cells treated with 10^{-5} and 10^{-11} M

glyphosate, respectively, for long-term as compared to the non-treated control, using RNU6B as an endogenous control (Figure 11). This experiment was performed only once (n=1), to be confirmed by more replicates.

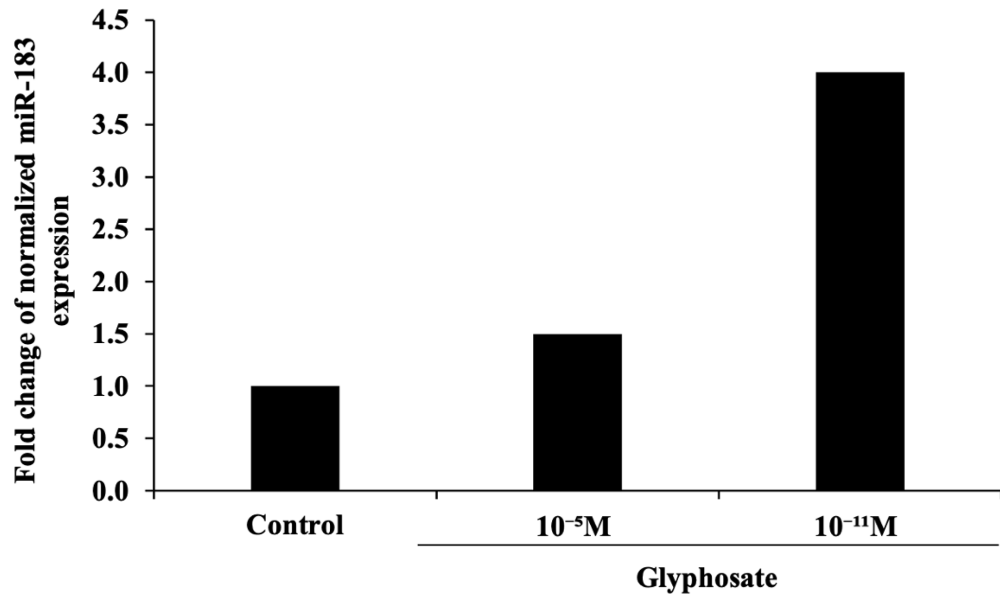

Figure 11: Long-term glyphosate exposure upregulates miR-183 expression in mammary epi-thelial SCp2 cells. miR-183 expression in glyphosate treated SCp2 cells was assessed by RT-qPCR using RNU6B as an endogenous control. The values de-picted in the histogram are the fold change of normalized miR-183 expression of only one experiment. (n=1; to be confirmed).

K. Upregulation of miR-183-5p, as well as downregulation of β-casein and differ-entiation of mammary epithelial cells are potential downstream targets of glyphosate use involved in pre-tumorigenic pathways.

Previous studies at our lab have revealed a link between miR-183 over-expression, breast cancer initiation, and the disruption of polarity in ductal epithelial cells as indi-cated by disrupted localization of polarity markers (Naser Al Deen et al. 2022). Another study showed that in tissue samples obtained from invasive lobular carcinoma (ILC) and lobular carcinoma in situ (LCIS), miR-183 was among the overexpressed miRNAs along with miR-182 and miR-375 identified in the epithelium (Naser Al Deen et al., 2019; Giricz et al., 2012). These findings suggested that miR-183 was associated with

the development of lobular neoplasia. Here, we sought to link glyphosate-induced miR-183 overexpression (Fig.11) with other intermediate genes involved in loss of differentiation and breast cancer initiation.

To identify the possible glyphosate-induced pathway that might be driving pre-tumorigenic phenotypes in SCp2 ER-positive, Ingenuity Pathway Analysis (IPA) was used. Some of the predicted (with high or moderate confidence) targets downstream of glyphosate that are implicated in breast cancer and ER-dependent are illustrated (Figure 12). Specifically, the superoxide dismutase type 1 (SOD1) enzyme, is indirectly inhibited by glyphosate, which leads to the direct activation of tumor protein P53 (TP53) transcriptional regulator, as SOD1 under normal conditions inhibits TP53. This in turn leads to the upregulation of miR-183-5p. Additionally, the upregulation of miR-183-5p is predicted to directly inhibit estrogen receptor 2 (ESR2). ESR2 is normally responsible for breast cancer gene 1 (BRCA1) activation, and thus the differentiation of mammary epithelial cells, and β-casein (CSN2) expression. Also, ESR2 under normal conditions inhibits breast cancer pathways. As such, when ESR2 is inhibited, the pathway for differentiation is inhibited, and breast cancer-related pathways are activated (to be added later).

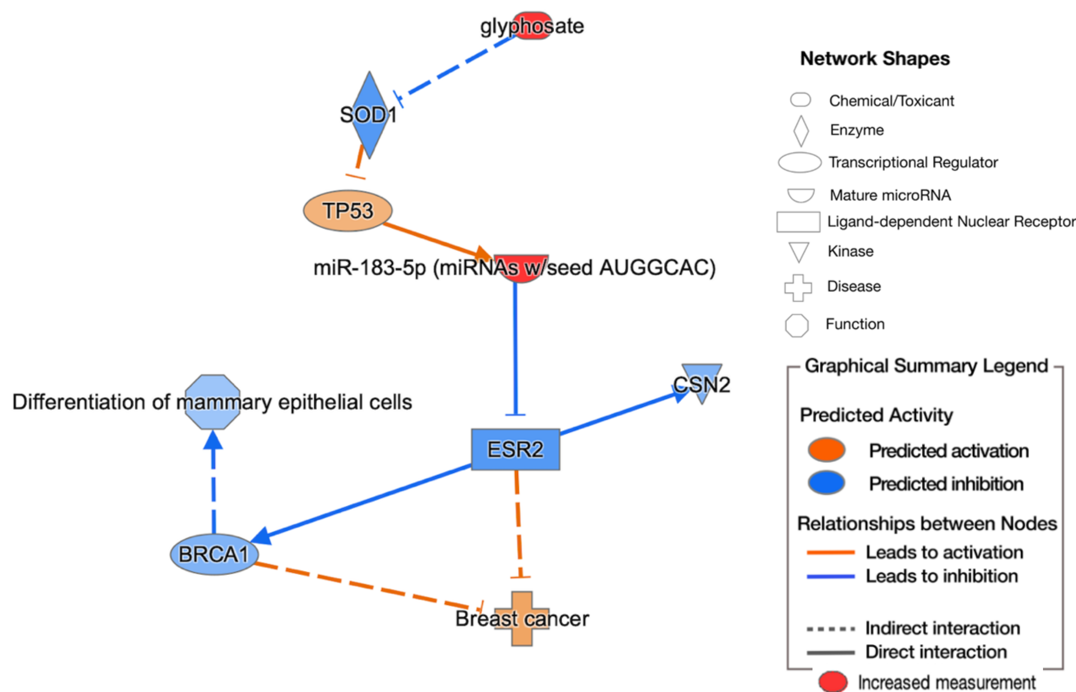

Figure 12: Predicted interactions between glyphosate and target genes involved in pre-tumorigenic pathways of ER-positive SCp2 cells. IPA was used to plot the predict-ed targets in a mechanistic network, using the Path Explorer which calculates the "Shortest Path.

Using the overlay MAP (Molecule Activity Predictor) in IPA, and glyphosate selected as "activated" (indicated in red), miR-183-5p is upregulated through the inhibition of SOD1 and the activation of TP53, and ESR2, thus inhibiting CSN2, are affected by glyphosate and involved in breast cancer pre-tumorigenic pathways and inhibition of differentiation of mammary epithelial cells.

The blue lines and molecules represent inhibition; orange lines and molecules represent activation. Solid lines imply direct relationships between gene products while dotted lines imply indirect interactions.

CHAPTER VI

DISCUSSION

Only 5 to 10% of breast cancers are thought to be directly brought on by hereditary genetic mutations, such as mutations in the BRCA1 and BRCA2 genes. The remaining 90% of breast cancer are associated with environmental contaminants and lifestyle factors that impact DNA and may result in genetic abnormalities in gene expression (American Cancer Society, 2021; Abdul et al., 2017; Anand et al., 2008). High intake of refined starches and sugars, smoking, infections, excess weight, alcohol, exposure to radiation and heavy metals, stress, lack of physical exercise, and the use of pesticides and herbicides such as glyphosate, among other causes, are environmental and lifestyle factors that have been implicated in breast cancer studies (Hiatt & Brody, 2018).

We report here the impact of glyphosate on tumor initiation events using lobular and ductal mammary epithelial 2D and 3D cell culture models with different ER status. Our primary focus was to evaluate the effects of glyphosate on the differentiation markers of these cell types. While numerous studies have identified potential risk factors associated with glyphosate use, the causal relationship between glyphosate exposure and breast cancer remains unclear (IARC Monographs, 2015; Williams et al., 2016). It is noteworthy that most countries have not placed a ban on the use of glyphosate (Klingelhöfer et al., 2021).

Glyphosate use and its link to cancer, including BC, have been the subject of contradictory reports. While some regulatory agencies state there is a lack of evidence connecting

glyphosate to cancer, other studies show evidence of genotoxic properties, increased oxidative stress, and estrogen pathway disruption, among other harmful effects (Mesnage et al., 2019). One study found that glyphosate exposure was associated with an increased risk of non-Hodgkin lymphoma (NHL) (Zhang et al., 2019). Another study reported an association between glyphosate exposure and an increased risk of breast cancer in postmenopausal women (Andreotti et al., 2018). However, in 2021, the European Union (EU) concluded that glyphosate poses minimal risk to human health and the environment and does not cause cancer, suggesting that there is no link between glyphosate and breast cancer. It is also important to note that the International Agency for Research on Cancer (IARC) classified glyphosate as a "probable human carcinogen" in 2015, while the US Environmental Protection Agency (EPA) and other regulatory agencies maintain that glyphosate is safe when used as directed. Interestingly, while it was thought that glyphosate acts through ER, therefore affecting cell lines that have tested positive for ER (Mesnage et al., 2017; Thongprakaisang et al., 2013), Stur et al. (2019) showed effects of glyphosate and its metabolite AMPA on both estrogen-positive and estrogen-negative breast cancer cell lines. The authors found that low doses of glyphosate were capable to alter canonical pathways, such as PI3K/Akt, ERK/MAPK, and JAK/STAT signaling pathways, in both cell lines.

We used well-characterized mammary epithelial cell lines; human-derived ER-negative ductal HMT-3522 S1 and mouse-derived ER-positive lobular SCp2 cells. 3D cell culture models have emerged as a promising tool in the fields of biology and medicine, with increasing use in recent years (Brancato et al., 2020). For example, according to Khafaga et al. (2022), a 3D cell culture model can preserve the genetic profile, heterogeneity, and structure of both cancerous and normal stromal cells in a perfect setting.

This suggests that a 3D cell culture model could potentially serve as a useful tool in cancer research and treatment by allowing for more accurate and representative modeling of the tumor microenvironment than 2D cultures. Koledova (2017) emphasizes that 3D cultures mimic important features of the *in vivo* environment, including cell-cell and cell-extracellular matrix interactions, which are particularly important in cancer biology.

S1 cells develop into well-differentiated glandular structures or acini that are marked by a polarity axis with apicolateral tight junctions when cultured in 3D with basement membrane components (Plachot & Lelièvre, 2004). Hence, we aimed to assess the effects of glyphosate on lumen formation and polarity in ductal epithelial cells. Furthermore, we sought to evaluate the differentiation marker of SCp2 lobular epithelial cells marked by the expression of the milk protein β-casein.

Data collected in our studies were obtained following long-term and short-term treatments with glyphosate at environmentally relevant and previously tested doses of 10^{-5} M and 10^{-11} M, which are lower than the concentrations detected in biological fluids such as milk, serum, and urine, as reported in previous studies (Steinborn et al., 2016; Yoshioka et al., 2011; Acquavella et al., 2004), and were similarly used by Sritana et al. (2018) in their study using human cholangiocarcinoma cell line (HuCCA-1). According to Wang et al. (2016), a degradation product of glyphosate, phosphonate AMPA, exhibited toxicity towards humans at a concentration of 2.5×10^{-4} M. According to the Environmental Protection Agency (EPA), residues of glyphosate on food are safe for consumers up to the established tolerances, which vary depending on the crop and range from 0.1 to 400 parts per million (ppm) (equivalent to 5.91×10^{-7} M to 2.36×10^{-4} M). A number of studies, including those by Thongprakaisang et al. (2013), Mesnage et al. (2017), and Sritana et al. (2018), have utilized a concentration of 10^{-11} M to examine

the impact of glyphosate on human cells. However, a separate investigation demonstrated that varying concentrations of glyphosate, ranging from 2 x 10^{-4} M to 6 x 10^{-3} M, elicited no discernible clastogenic impact in cultures of peripheral human lymphocytes (Mañas et al., 2009). As for the half-life of glyphosate, Bento et al. (2016) reported that glyphosate remains in the soil between 1 and 197 days, whereas Gandhi et al. (2021) indicated that the period taken to degrade 50% of glyphosate in water is less than 14 days in aerobic environments and around 14-22 days in anaerobic environments. Because the half-life of glyphosate varies depending on the condition, we referred to studies that have used *in vitro* cell cultures to study pesticides. Imidacloprid, a neonicotinoid insecticide, was studied *in vitro* using human liver cells for a period of 10 days (Özdemir et al., 2018), a duration that we utilized as short-term. Whereas, Atrazine, an herbicide, was studied *in vitro* using human prostate cancer cells for a period of 21 days (Huang et al., 2015), a duration that we utilized as long-term. In addition, we employed long-term treatment to examine the persistence of tumor initiation outcomes subsequent to transfer.

We first evaluated the effect of 10^{-5} and 10^{-11} M glyphosate on S1 cell counts. In our study, 10^{-5} M glyphosate was the highest concentration with no evident effect on cell viability and proliferation (unpublished data), where cells did not grow consistently with the control untreated cells after day 8 in culture pre- and post-transfer. This indicates that a higher concentration of 10^{-5} M glyphosate treatment for a prolonged period may affect cell growth rate. However, S1 cells that were exposed to a concentration of 10^{-11} M glyphosate increased in a similar manner to untreated control cells during the first and second passages, indicating no effect on cell growth.

We next looked into tumor initiation events triggered by glyphosate in non-tumorigenic S1 cells. We show that both long-term and short-term 10^{-5} and 10^{-11} M glyphosate treatments enhanced invasive capacities in non-tumorigenic human S1 cells. This observation aligns with earlier research wherein the upregulation of miR-182-5p and glyphosate treatment in nontumorigenic breast MCF10A cells show invasive patterns similar to MCF7 and MDA-MB-231 breast cancer cell lines in 2D, as demonstrated by Duforestel et al. (2019).

We additionally show that short-term glyphosate treatment disrupts lumen assembly in 3D cultures of S1 cells when treated pre-lumen assembly alone (before day 7), as well as pre- and post-lumen assembly, but not post-lumen assembly (after day 7). Likewise, long-term glyphosate treatment pre- and post-lumen assembly disrupt lumen formation. Glyphosate may have the ability to disturb the process of lumen formation and the polarized phenotype before it is established, which in turn may affect cell junctions. Indeed, previous studies have shown that treatment with 18-α GA downregulated the expression of connexin 43 (Cx43) which led to decreased GJIC, and disrupted lumen assembly (Bazzoun et al., 2019; El Sabban et al., 2003), suggesting that glyphosate may have a similar effect in disrupting lumen assembly through modulating gap junctional activity. However, glyphosate does not interfere with lumen assembly after it has been formed. Furthermore, another study reported that gap junctions are potential targets for new insecticides, as gap junction inhibitors elicit toxic effects on adult female mosquitoes (Calkins & Piermarini, 2015). Noteworthy is that gap junctions and Cx43 expression were altered in response to a large number of environmental xenobiotics, including herbicides, pesticides, and heavy metals. For example, Pointis et al. (2011) found that

certain herbicides and pesticides can alter gap junction-mediated communication in Sertoli cells, which are important for spermatogenesis. Meanwhile, Fiorini et al. (2004) showed that exposure to lead, cadmium, and mercury can lead to decreased expression of Cx43 in the liver, which may contribute to liver dysfunction.

To control cell development and proliferation, polarity proteins Cx43 and β-catenin interact, while Cx43 and cadherins appear to control β-catenin signaling by sequestering it at the membrane (Spagnol et al., 2018; Talhouk et al., 2013; Talhouk et al., 2008). Additionally, binding of E- or N-cadherins (E- or N-) to β-catenin is essential for the suppression of cell proliferation (Gottardi, Wong, & Gumbiner, 2001; Kamei, Toyofuku, & Hori, 2003). As a result, one of the indicators of junctional disassembly is the relocalization of β-catenin to the nucleus and activation of the canonical Wnt/β-catenin pathway (Prasad et al., 2008; Jönsson et al., 2000). Previously, we found that Cx43-shRNA S1 cells, which serve as pre-tumorigenic models for BC, lacked apical polarity and showed disrupted lumen assembly in 3D cultures, and activated invasion pathways (Fostok et al., 2019; Bazzoun et al., 2016). Although our data show that glyphosate treatment did not alter the expression levels of Cx43 and β-catenin, we postulate that the mislocalization of Cx43 and β-catenin could induce the loss of lumen formation and enhance the invasive potential as previously reported by Bazzoun et al., 2016.

In our study, we indeed show the relocalization of β-catenin from an apicolateral to a basolateral distribution pattern upon treatment with long-term and short-term 10^{-5} and 10^{-11} M glyphosate. Apicobasal polarity is important for controlling epithelial processes such as proliferation, migration, apoptosis, morphology, and differentiation (Chatterjee & McCaffrey, 2014). Studies have shown that loss of apical polarity can lead to cell cy-

cle entry in S1 acini, but this alone is not sufficient to increase proliferation (Chandramouly et al., 2007). Additionally, in endothelial cells, altered distribution of β-catenin in the membrane is linked to weakened cell-to-cell connections, gaps between cells, and increased permeability (Scholz et al., 2004). Thus, as a result of weakened cell-to-cell contact, the relocalization of β-catenin in S1 cells may prompt invasion.

To assess the effect of glyphosate on tumor initiation events and differentiation pattern of other cell types, we used lobular nontumorigenic mouse derived SCp2 cells. 10^{-5} and 10^{-11} M glyphosate did not exhibit a cytotoxic effect on SCp2 cells. Interestingly, our results indicate that long-term treatment with 10^{-11} M glyphosate triggered an enhanced invasive phenotype, but not with the higher concentration of 10^{-5} M. This can be possibly explained by the upregulation of MMP-9 which is more evident upon 10^{-11} M glyphosate treatment. In fact, MMP9 protein expression was found to be associated with high tumor grade and Nottingham Prognostic Index, both of which are indicators of more aggressive breast cancer (Izdebska et al., 2021). In addition, MMP9 overexpression was more frequent in patients with larger tumor sizes (Jiang and Li, 2021), and is also classified as a tumor biomarker and prognostic factor, suggesting that it may have clinical value in predicting disease progression (Joseph et al., 2020). Therefore, this might be indicative of a stronger effect of 10^{-11} M glyphosate compared to 10^{-5} M in tumor progression, marked by higher levels of MMP9. In fact, previous studies have shown epigenetic changes upon low concentrations of the endocrine-disrupting chemical Bisphenol-A (BPA) even at low concentrations as opposed to high concentrations (Longo et al., 2020; Vandenberg et al., 2012). Moreover, Aouad et al. (2017) found that the synthetic retinoid ST1926 exhibits potent antitumor activities in both 2D and 3D

breast cancer cell models, with the strongest activity observed at low concentrations, suggesting that chemicals and drugs work in a dose-dependent manner.

This may also be indicative of the presence of tissue inhibitors of metalloproteinases (TIMPs) in the 10^{-5} M glyphosate treated cells to counterbalance the low levels of MMP-9. In 10^{-11} M treated cells in contrast, levels of TIMPs may be similar to those in 10^{-5} M. In fact, TIMP-1 has been found to have antiapoptotic properties in MCF10A cells. Specifically, overexpression of TIMP-1 has been shown to inhibit apoptosis following the loss of cell adhesion, or anoikis, independent of its ability to stabilize cell-matrix interactions. Additionally, TIMP-1 overexpression has been associated with the constitutive activation of focal adhesion kinase (FAK), a signaling molecule critical for the cell survival pathway, in MCF10A cells (Li et al., 1999). Talhouk et al. (1992) showed that involuting mammary glands that received TIMPs implants maintained high levels of ß -casein and delayed alveolar regression, and apoptosis (Lund et al., 1996), suggesting that the balance of ECM-degrading proteinases and their inhibitors regulates the organization of the basement membrane and the tissue-specific function of the mammary gland. Indeed, our data show that ß-casein expression in SCp2 cells is downregulated upon glyphosate exposure, therefore affecting the differentiation of SCp2 cells. We have previously shown that ß-casein is dependent on gap junctional assembly (Talhouk et al., 2008), therefore we speculate that similarly to S1 cells, glyphosate may be targeting junctional assembly through ß-casein downregulation.

Glyphosate has been linked to miRNA dysregulation in various models. For instance, glyphosate exposure leads to the upregulation of 55 and downregulation of 19 miRNA in the pre-frontal cortex of post-natal mice offspring (Ji et al., 2018). In the study by Duforestel et al. (2019), glyphosate treatment in combination with miR-182-5p

overexpression led to tumor initiation in mice and the breast epithelial MCF10A cell line in 2D. However, in our study, S1 cells showed tumor-initiated phenotype marked by disrupted lumen assembly in 3D. Therefore, it is plausible that the environment *in vivo* may have played a suppressive role, where other molecules and factors present in the tumor microenvironment may interact differently with glyphosate-induced pathways. For example, Gudjonsson et al. (2002) found that normal myoepithelial cells were able to interact with luminal epithelial cells and promote proper polarity and basement membrane deposition, whereas tumor-derived myoepithelial cells showed a lack of ability to interact with luminal epithelial cells and interfere with lumen formation. Additionally, Truffi et al., (2020) reviewed that fibroblasts can promote tumor growth and angiogenesis, but they can also produce factors that inhibit tumor growth and promote an anti-tumor immune response. One study analyzed the effects of different concentrations of Roundup (glyphosate) on the hatching of miracidia, mortality and excystment rate of metacercariae, parasitic load, and egg production *in vitro* using human and animal cells, and *in vivo* using snails, fish, amphibians and rats. The study found that the herbicide concentrations affected the specimens differently *in vitro* and *in vivo*, indicating that the *in vitro* and *in vivo* treatments should not be considered interchangeable (Monte et al., 2016).

MiR-183 is shown to be involved in epithelial polarity pathways and is upregulated upon Cx43 loss. It is the most upregulated miRNA in early-stage breast cancer patient cohort (and matched US patients) (Nassar et al., 2017) and its upregulation conferred with the increased risk of cancer progression in the 3D culture model recapitulating tumor-initiation phenotypes seen upon Cx43 loss (Naser Al Deen et al., 2022). MiR-183 was confirmed among a biomarker panel of miRNAs whose upregulation predicted

the tumor progression of lobular neoplasia from an *in situ* to a malignant transformation brought by loss of cellular polarity and acquisition of a hyperplastic phenotypes (Giricz et al., 2012). Additionally, preliminary data from our lab show that SCp2 cells under non-differentiation-permissive conditions (lack of basement membrane), the gap junctional complex is non-functional (Talhouk et al., 2008), and miR-183 is upregulated (unpublished data). We therefore aimed to investigate whether glyphosate treatment increases miR-183-5p expression leading to the downregulation of tumor suppressing signaling pathways involved in cell communication and differentiation in SCp2 cells. Our preliminary data indicate an increase in miR-183 expression upon treatment with 10^{-11} M glyphosate. Ongoing research in our lab show that Integrin beta 1 (ITGB1) is an experimentally validated target of miR-183-5p which highlights this miRNA's potential role in disrupting the integrin pathway responsible for alveolar mammary epithelial differentiation (unpublished data).

The potential mechanism of action of glyphosate in estrogen-dependent cell lines was investigated using Ingenuity Pathway Analysis (IPA), which predicts downstream effects, identifies new targets, and candidate biomarkers (QIAGEN, Ingenuity Pathway Analysis). One of the most useful features of IPA is the Mechanistic Networks, which can automatically generate plausible signaling cascades, describing potential mechanisms of action that lead to observed gene expression changes caused by chemicals or diseases. Glyphosate targets were chosen for high confidence and relevance to our study, assuming that miR-183 is upregulated based on previous studies (Naser Al Deen et al., 2022). IPA showed that glyphosate's downstream target was the upregulation of miR-183, which is consistent with our preliminary results. Studies by Cheng et al. (2016) and Giricz et al. (2012) showed that miR-183-5p is overexpressed in

breast cancer patients, leading to increased cell proliferation and decreased apoptosis. We therefore hypothesize that, through acting as an oncomiR, miR-183-5p overexpression is likely driving the pre-tumorigenic phenotypes triggered by glyphosate in the breast epithelium. Moreover, IPA revealed that glyphosate is linked to miR-183-5p overexpression through downregulation of Superoxide dismutase (SOD1), which upregulates tumor protein p53 (TP53). SOD1 is an essential enzyme that protects cells from oxidative stress by converting superoxide radicals into hydrogen peroxide and oxygen. Recent studies have indicated that SOD1 also regulates cellular processes like apoptosis, protein synthesis, and signal transduction (Eleutherio et al., 2021). Glyphosate, as reported by Ma et al. (2019), has the ability to suppress SOD's functions, leading to an overproduction of lipid peroxides in adult fish. Moreover, Rao et al. (2009) indicated that nuclear SOD1 interacts with ERα, which is critical for the successful activation of estrogen-responsive genes. In light of previous research on lizard liver, glyphosate could cause SOD1 to translocate into the nucleus, activating estrogen-responsive genes such as VTG (Rao et al., 2009). Our IPA analyses also show that one of the pathways through which miR-183-5p downregulates beta-casein (CSN2) and BRCA1, and causes the onset of breast cancer, is through the downregulation of ESR2 (Estrogen Receptor Beta, Erβ). Interestingly, it was shown that the expression of ERα and ERβ are substantially elevated after being subjected to glyphosate treatments (Verderame and Scudiero, 2019). Indeed, in an *in vitro* setting using human breast cancer cells, it was demonstrated that glyphosate's action mediated by ER was hindered by ICI 182780, an estrogen antagonist, which inhibited the ERE transcription activity induced by glyphosate (Thongprakaisang et al., 2013).

In summary, our results demonstrate that glyphosate treatment induces tumor initiation events such as enhanced invasion, MMP-9 release, and upregulation of miR-183. We propose that glyphosate-induced tumor initiation events drive mammary epithelial cells to a less differentiated state *in vitr*o, evident by the loss of apical polarity and normal acinar morphology in ductular S1 cells, and the downregulation of ß-casein in lobular SCp2 cells. In future experiments, we will work on deciphering the underlying molecular mechanisms that drive the tumorigenic changes in our 3D systems, potentially through analysis of the proposed signaling cascade or gene expression profiles of ER-positive cells.

To our knowledge, our study is unique in testing the effect of glyphosate treatment on the differentiation of non-tumorigenic human-derived ductal S1 and mouse-derived lobular SCp2 mammary epithelial cells under differentiation-permissive conditions (presence of basement membrane components). Our findings highlight its effect on inducing loss of differentiation and breast cancer initiation events in human and mouse-derived non-tumorigenic breast epithelial cells, *in vitro*. We therefore speculate that glyphosate may affect cell-ECM interaction and/or GJIC, and therefore disturbing normal differentiation of mammary epithelial cells, and inducing tumor initiation events.

In light of our findings, it is recommended to exercise caution when using glyphosate-based herbicides, particularly for individuals who may encounter highly concentrated solutions of these herbicides, such as farmers. It is important to monitor the use of glyphosate by farmers to prevent excessive use, as it is a leading herbicide used for the management of invasive and noxious weeds in various settings, including public land, residential areas, pastures, and forests (EPA, 2023), as it is ending up in our

food chain (Bayer Crop Science, 2022). Furthermore, it is recommended that additional experimental and observational studies be conducted to enable regulatory agencies and health officials to make informed decisions concerning the implementation of regulations and restrictions on glyphosate-based herbicides.

CHAPTER VII

CONCLUSION

In conclusion, our study is unique in testing the effect of glyphosate on lobular and ductal mammary epithelial cells, with focus on differentiation and tumor initiation events. Our findings highlight glyphosate effects on inducing loss of differentiation and breast cancer initiation events in human and mice nontumorigenic breast epithelial cells, *in vitro*. We therefore speculate that glyphosate may affect cell-ECM interaction or GJIC, and therefore disturbing normal development and differentiation of the mammary gland. However, this interpretation needs further investigation in our future studies. This could include exposing animals to glyphosate through the common exposure routes such as ingestion and inhalation, and examining the resulting changes in mammary gland development, gene expression, and differentiation. Avoiding the extensive use of glyphosate and the improper application practices to prevent adverse effects related to glyphosate use, in addition, limiting DNA hypomethylation by using and developing inhibitor of TET-3 for example, would be interesting directions to prevent tumor incidence in the case of glyphosate exposure (Duforestel et al., 2019). The epigenetic pathway that leads to global DNA hypomethylation and GJ functionality in mammary cells are important aspects to further study and assess glyphosate risk on breast cancer.

BIBLIOGRAPHY

Abbott, A. (2003). Biology's new dimension. Nature, 424, 870-872. https://doi.org/10.1038/424870a

Abdul, Q.A., Yu, B.P., Chung, H.Y. *et al.* Epigenetic modifications of gene expression by lifestyle and environment. *Arch. Pharm. Res.* **40**, 1219–1237 (2017). https://doi.org/10.1007/s12272-017-0973-3

Abraham, V., Chou, M. L., George, P., Pooler, P., Zaman, A., Savani, R. C., & Koval, M. (2001). Heterocellular gap junctional communication between alveolar epithelial cells. *American journal of physiology. Lung cellular and molecular physiology*, *280*(6), L1085–L1093. https://doi.org/10.1152/ajplung.2001.280.6.L1085

Acquavella, J. F., Alexander, B. H., Mandel, J. S., Gustin, C., Baker, B., Chapman, P., & Bleeke, M. (2004). Glyphosate biomonitoring for farmers and their families: results from the Farm Family Exposure Study. *Environmental health perspectives*, *112*(3), 321–326. https://doi.org/10.1289/ehp.6667

Abraham, V., Chou, M. L., George, P., Pooler, P., Zaman, A., Savani, R. C., & Koval, M. (2001). Heterocellular gap junctional communication between alveolar epithelial cells. *American journal of physiology. Lung cellular and molecular physiology*, *280*(6), L1085–L1093. https://doi.org/10.1152/ajplung.2001.280.6.L1085

Alcaraz, J., Xu, R., Mori, H., Nelson, C. M., Mroue, R., Spencer, V. A., Brownfield, D., Radisky, D. C., Bustamante, C., & Bissell, M. J. (2008). Laminin and biomimetic extra-cellular elasticity enhance functional differentiation in mammary epithelia. *The EMBO journal*, *27*(21), 2829–2838. https://doi.org/10.1038/emboj.2008.206

Anand, P., Kunnumakara, A.B., Sundaram, C. *et al.* Cancer is a Preventable Disease that Requires Major Lifestyle Changes. *Pharm Res* **25**, 2097–2116 (2008). https://doi.org/10.1007/s11095-008-9661-9

Anderson, J. M., & Van Itallie, C. M. (2009). Physiology and function of the tight junction. *Cold Spring Harbor perspectives in biology*, *1*(2), a002584. https://doi.org/10.1101/cshperspect.a002584

Andreotti, G., Koutros, S., Hofmann, J. N., Sandler, D. P., Lubin, J. H., Lynch, C. F., Lerro, C. C., De Roos, A. J., Parks, C. G., Alavanja, M. C., Silverman, D. T., & Beane Freeman, L. E. (2018). Glyphosate Use and Cancer Incidence in the Agricultural Health Study. *Journal of the National Cancer Institute*, *110*(5), 509–516. https://doi.org/10.1093/jnci/djx233

Aouad, P. , Saikali, M. , Abdel-Samad, R. , Fostok, S. , El-Houjeiri, L. , Pisano, C. , Talhouk, R. & Darwiche, N. (2017). Antitumor activities of the synthetic retinoid ST1926 in two-dimensional and three-dimensional human breast cancer models. *Anti-Cancer Drugs, 28* (7), 757-770. doi: 10.1097/CAD.0000000000000511

Asiaf, A., Ahmad, S. T., Malik, A. A., Aziz, S. A., & Zargar, M. A. (2019). Association of Protein Expression and Methylation of DAPK1 with Clinicopathological Features in Invasive Ductal Carcinoma Patients from Kashmir. *Asian Pacific journal of cancer prevention : APJCP*, *20*(3), 839–848. https://doi.org/10.31557/APJCP.2019.20.3.839

Assémat, E., Bazellières, E., Pallesi-Pocachard, E., Le Bivic, A., & Massey-Harroche, D. (2008). Polarity complex proteins. *Biochimica et biophysica acta*, *1778*(3), 614–630. https://doi.org/10.1016/j.bbamem.2007.08.029

Aune, D., Giovannucci, E., Boffetta, P., Fadnes, L. T., Keum, N., Norat, T., Greenwood, D. C., Riboli, E., Vatten, L. J., & Tonstad, S. (2017). Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality-a systematic review and dose-response meta-analysis of prospective studies. *International journal of epidemiology*, *46*(3), 1029–1056. https://doi.org/10.1093/ije/dyw319

Bae, Y. K., Brown, A., Garrett, E., Bornman, D., Fackler, M. J., Sukumar, S., Herman, J. G., & Gabrielson, E. (2004). Hypermethylation in histologically distinct classes of breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*, *10*(18 Pt 1), 5998–6005. https://doi.org/10.1158/1078-0432.CCR-04-0667

Banerjee D. (2016). Connexin's Connection in Breast Cancer Growth and Progression. *International journal of cell biology*, *2016*, 9025905. https://doi.org/10.1155/2016/9025905

Bazzoun, D., Adissu, H. A., Wang, L., Urazaev, A., Tenvooren, I., Fostok, S. F., Chittiboyina, S., Sturgis, J., Hodges, K., Chandramouly, G., Vidi, P. A., Talhouk, R. S., & Lelièvre, S. A. (2019). Connexin 43 maintains tissue polarity and regulates mitotic spindle orientation in the breast epithelium. *Journal of cell science*, *132*(10), jcs223313. https://doi.org/10.1242/jcs.223313

Bazzoun, D., Adissu, H. A., Wang, L., Urazaev, A., Tenvooren, I., Fostok, S. F., Chittiboyina, S., Sturgis, J., Hodges, K., Chandramouly, G., Vidi, P. A., Talhouk, R. S., & Lelièvre, S. A. (2019). Connexin 43 maintains tissue polarity and regulates mitotic spindle orientation in the breast epithelium. *Journal of cell science*, *132*(10), jcs223313. https://doi.org/10.1242/jcs.223313

Behravan, H., Hartikainen, J.M., Tengström, M. *et al.* Predicting breast cancer risk using interacting genetic and demographic factors and machine learning. *Sci Rep* **10**, 11044 (2020). https://doi.org/10.1038/s41598-020-66907-9

Ben-Salem, S., Venkadakrishnan, V. B., & Heemers, H. V. (2021). Novel insights in cell cycle dysregulation during prostate cancer progression. *Endocrine-related cancer*, *28*(6), R141–R155. https://doi.org/10.1530/ERC-20-0517

Bento, C. P. M., Yang, X., Gort, G., Xue, S., van Dam, R., Zomer, P., Mol, H. G. J., Ritsema, C. J., & Geissen, V. (2016). Persistence of glyphosate and aminomethylphosphonic acid in loess soil under different combinations of temperature, soil moisture and light/darkness. *The Science of the total environment*, *572*, 301–311. https://doi.org/10.1016/j.scitotenv.2016.07.215

Bergstralh, D. T., Haack, T., & St Johnston, D. (2013). Epithelial polarity and spindle orientation: intersecting pathways. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *368*(1629), 20130291. https://doi.org/10.1098/rstb.2013.0291

Bernardo, G. M., Bebek, G., Ginther, C. L., Sizemore, S. T., Lozada, K. L., Miedler, J. D., Anderson, L. A., Godwin, A. K., Abdul-Karim, F. W., Slamon, D. J., & Keri, R. A. (2013). FOXA1 represses the molecular phenotype of basal breast cancer cells. *Oncogene*, *32*(5), 554–563. https://doi.org/10.1038/onc.2012.62

Beroukhim, R., Mermel, C. H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., Barretina, J., Boehm, J. S., Dobson, J., Urashima, M., Mc Henry, K. T., Pinchback, R. M., Ligon, A. H., Cho, Y. J., Haery, L., Greulich, H., Reich, M., Winckler, W., Lawrence, M. S., Weir, B. A., … Meyerson, M. (2010). The landscape of somatic copy-number alteration across human cancers. *Nature*, *463*(7283), 899–905. https://doi.org/10.1038/nature08822

Bhatia, S., Monkman, J., Blick, T., Pinto, C., Waltham, M., Nagaraj, S. H., & Thompson, E. W. (2019). Interrogation of Phenotypic Plasticity between Epithelial and Mesenchymal States in Breast Cancer. *Journal of clinical medicine*, *8*(6), 893. https://doi.org/10.3390/jcm8060893

Biedermann, K., Vogelsang, H., Becker, I., Plaschke, S., Siewert, J. R., Höfler, H., & Keller, G. (2005). Desmoglein 2 is expressed abnormally rather than mutated in familial and sporadic gastric cancer. *The Journal of pathology*, *207*(2), 199–206. DOI: 10.1002/path.1821

Biswas, S. K., Banerjee, S., Baker, G. W., Kuo, C. Y., & Chowdhury, I. (2022). The Mammary Gland: Basic Structure and Molecular Signaling during Development. *International journal of molecular sciences*, *23*(7), 3883. https://doi.org/10.3390/ijms23073883

Blaschke, R. J., Howlett, A. R., Desprez, P. Y., Petersen, O. W., & Bissell, M. J. (1994). Cell differentiation by extracellular matrix components. *Methods in enzymology*, *245*, 535–556. https://doi.org/10.1016/0076-6879(94)45027-7

Bøhn, T., Cuhra, M., Traavik, T., Sanden, M., Fagan, J., & Primicerio, R. (2014). Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans. *Food chemistry*, *153*, 207–215. https://doi.org/10.1016/j.foodchem.2013.12.054

Bøhn, T., Cuhra, M., Traavik, T., Sanden, M., Fagan, J., & Primicerio, R. (2014). Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans. *Food chemistry*, *153*, 207–215. https://doi.org/10.1016/j.foodchem.2013.12.054

Boice J. D., Jr (2012). Radiation epidemiology: a perspective on Fukushima. *Journal of radiological protection : official journal of the Society for Radiological Protection*, *32*(1), N33–N40. https://doi.org/10.1088/0952-4746/32/1/N33

Boice, J. D., Jr, Preston, D., Davis, F. G., & Monson, R. R. (1991). Frequent chest X-ray fluoroscopy and breast cancer incidence among tuberculosis patients in Massachusetts. *Radiation research*, *125*(2), 214–222.

Borcherding, N., Cole, K., Kluz, P., Jorgensen, M., Kolb, R., Bellizzi, A., & Zhang, W. (2018). Re-Evaluating E-Cadherin and β-Catenin: A Pan-Cancer Proteomic Approach with an Emphasis on Breast Cancer. *The American journal of pathology*, *188*(8), 1910–1920. https://doi.org/10.1016/j.ajpath.2018.05.003

Boyd, N. F., Stone, J., Vogt, K. N., Connelly, B. S., Martin, L. J., & Minkin, S. (2003). Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature. *British journal of cancer*, *89*(9), 1672–1685. https://doi.org/10.1038/sj.bjc.6601314

Brancato, V., Oliveira, J. M., Correlo, V. M., Reis, R. L., & Kundu, S. C. (2020). Could 3D models of cancer enhance drug screening?. *Biomaterials*, *232*, 119744. https://doi.org/10.1016/j.biomaterials.2019.119744

Brennan, K., Offiah, G., McSherry, E. A., & Hopkins, A. M. (2010). Tight junctions: a barrier to the initiation and progression of breast cancer?. *Journal of biomedicine & biotechnology*, *2010*, 460607. https://doi.org/10.1155/2010/460607

Briand, P., Petersen, O.W. & Van Deurs, B. A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. *In Vitro Cell Dev Biol* **23**, 181–188 (1987). https://doi.org/10.1007/BF02623578

Brisken, C., & O'Malley, B. (2010). Hormone action in the mammary gland. *Cold Spring Harbor perspectives in biology*, *2*(12), a003178. https://doi.org/10.1101/cshperspect.a003178

Brody, J. G., Moysich, K. B., Humblet, O., Attfield, K. R., Beehler, G. P., & Rudel, R. A. (2007). Environmental pollutants and breast cancer: epidemiologic studies. *Cancer*, *109*(12 Suppl), 2667–2711. https://doi.org/10.1002/cncr.22655

Calkins, T. L., & Piermarini, P. M. (2015). Pharmacological and Genetic Evidence for Gap Junctions as Potential New Insecticide Targets in the Yellow Fever Mosquito, Aedes aegypti. *PloS one*, *10*(9), e0137084. https://doi.org/10.1371/journal.pone.0137084

Chandramouly, G., Abad, P. C., Knowles, D. W., & Lelièvre, S. A. (2007). The control of tissue architecture over nuclear organization is crucial for epithelial cell fate. *Journal of cell science*, *120*(Pt 9), 1596–1606. https://doi.org/10.1242/jcs.03439

Chanson, L., Brownfield, D., Garbe, J. C., Kuhn, I., Stampfer, M. R., Bissell, M. J., & LaBarge, M. A. (2011). Self-organization is a dynamic and lineage-intrinsic property of mammary epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(8), 3264–3269. https://doi.org/10.1073/pnas.1019556108

Chatterjee, S. J., & McCaffrey, L. (2014). Emerging role of cell polarity proteins in breast cancer progression and metastasis. *Breast cancer (Dove Medical Press)*, *6*, 15–27. https://doi.org/10.2147/BCTT.S43764

Chen, X., & Yan, G. Y. (2013). Novel human lncRNA-disease association inference based on lncRNA expression profiles. *Bioinformatics (Oxford, England)*, *29*(20), 2617–2624. https://doi.org/10.1093/bioinformatics/btt426

Cheng, Y., Xiang, G., Meng, Y., & Dong, R. (2016). MiRNA-183-5p promotes cell proliferation and inhibits apoptosis in human breast cancer by targeting the PDCD4. *Reproductive biology*, *16*(3), 225–233. https://doi.org/10.1016/j.repbio.2016.07.002

Chiba, H., Osanai, M., Murata, M., Kojima, T., & Sawada, N. (2008). Transmembrane proteins of tight junctions. *Biochimica et biophysica acta*, *1778*(3), 588–600. https://doi.org/10.1016/j.bbamem.2007.08.017

Chua, A. C., Hodson, L. J., Moldenhauer, L. M., Robertson, S. A., & Ingman, W. V. (2010). Dual roles for macrophages in ovarian cycle-associated development and remodelling of the mammary gland epithelium. *Development, 137*(24), 4229- 4238. DOI: 10.1242/dev.059261

Ciriello, G., Gatza, M. L., Beck, A. H., Wilkerson, M. D., Rhie, S. K., Pastore, A., Zhang, H., McLellan, M., Yau, C., Kandoth, C., Bowlby, R., Shen, H., Hayat, S., Fieldhouse, R., Lester, S. C., Tse, G. M., Factor, R. E., Collins, L. C., Allison, K. H., Chen, Y., & Perou, C. M. (2015). Comprehensive molecular portraits of invasive lobular breast cancer. *Cell, 163*(2), 506–519. DOI: 10.1016/j.cell.2015.09.033

Daniel, J. M., & Reynolds, A. B. (1995). The tyrosine kinase substrate p120cas binds directly to E-cadherin but not to the adenomatous polyposis coli protein or alpha-catenin. *Molecular and cellular biology, 15*(9), 4819–4824. DOI: 10.1128/MCB.15.9.4819

Debnath, J., Muthuswamy, S. K., & Brugge, J. S. (2003). Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods (San Diego, Calif.)*, *30*(3), 256–268. https://doi.org/10.1016/s1046-2023(03)00032-x

Deng, T., Lyon, C. J., Bergin, S., Caligiuri, M. A., & Hsueh, W. A. (2016). Obesity, Inflammation, and Cancer. *Annual review of pathology*, *11*, 421–449. https://doi.org/10.1146/annurev-pathol-012615-044359

Deugnier, M. A., Faraldo, M. M., Teulière, J., Thiery, J. P., Medina, D., & Glukhova, M. A. (2006). Isolation of mouse mammary epithelial progenitor cells with basal characteristics from the Comma-Dbeta cell line. *Developmental biology*, *293*(2), 414–425. https://doi.org/10.1016/j.ydbio.2006.02.007

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, R. T., & Gore, A. C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine reviews*, *30*(4), 293–342. https://doi.org/10.1210/er.2009-0002

Donato, F., Pira, E., Ciocan, C., & Boffetta, P. (2020). Exposure to glyphosate and risk of non-Hodgkin lymphoma and multiple myeloma: an updated meta-analysis. *La Medicina del lavoro*, *111*(1), 63–73. https://doi.org/10.23749/mdl.v111i1.8967

Dong, S., Terasaka, S., & Kiyama, R. (2011). Bisphenol A induces a rapid activation of Erk1/2 through GPR30 in human breast cancer cells. *Environmental pollution (Barking, Essex : 1987)*, *159*(1), 212–218. https://doi.org/10.1016/j.envpol.2010.09.004

Doppler, W., Groner, B., & Ball, R. K. (1989). Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat beta-casein gene promoter constructs in a mammary epithelial cell line. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(1), 104–108. https://doi.org/10.1073/pnas.86.1.104

Dow, L. E., & Humbert, P. O. (2007). Polarity regulators and the control of epithelial architecture, cell migration, and tumorigenesis. *International review of cytology*, *262*, 253–302. https://doi.org/10.1016/S0074-7696(07)62006-3

Dravis, C., Spike, B. T., Harrell, J. C., Johns, C., Trejo, C. L., Southard-Smith, E. M., Perou, C. M., & Wahl, G. M. (2015). Sox10 Regulates Stem/Progenitor and Mesenchymal Cell States in Mammary Epithelial Cells. *Cell reports*, *12*(12), 2035–2048. https://doi.org/10.1016/j.celrep.2015.08.040

Du, Q., & Macara, I. G. (2004). Mammalian Pins is a conformational switch that links NuMA to heterotrimeric G proteins. *Cell*, *119*(4), 503–516. https://doi.org/10.1016/j.cell.2004.10.028

Duforestel, M., Nadaradjane, A., Bougras-Cartron, G., Briand, J., Olivier, C., Frenel, J. S., Vallette, F. M., Lelièvre, S. A., & Cartron, P. F. (2019). Glyphosate Primes Mammary Cells for Tumorigenesis by Reprogramming the Epigenome in a TET3-Dependent Manner. *Frontiers in genetics*, *10*, 885. https://doi.org/10.3389/fgene.2019.00885

Durgan, J., Kaji, N., Jin, D., & Hall, A. (2011). Par6B and atypical PKC regulate mitotic spindle orientation during epithelial morphogenesis. *The Journal of biological chemistry*, *286*(14), 12461–12474. https://doi.org/10.1074/jbc.M110.174235

Eichelser, C., Flesch-Janys, D., Chang-Claude, J., Pantel, K., & Schwarzenbach, H. (2013). Deregulated serum concentrations of circulating cell-free microRNAs miR-17, miR-34a, miR-155, and miR-373 in human breast cancer development and progression. *Clinical chemistry*, *59*(10), 1489–1496. https://doi.org/10.1373/clinchem.2013.205161

El-Sabban, M. E., Abi-Mosleh, L. F., & Talhouk, R. S. (2003). Developmental regulation of gap junctions and their role in mammary epithelial cell differentiation. *Journal of mammary gland biology and neoplasia*, *8*(4), 463–473. https://doi.org/10.1023/B:JOMG.0000017432.04930.76

Eleutherio, E. C. A., Silva Magalhães, R. S., de Araújo Brasil, A., Monteiro Neto, J. R., & de Holanda Paranhos, L. (2021). SOD1, more than just an antioxidant. *Archives of biochemistry and biophysics*, *697*, 108701. https://doi.org/10.1016/j.abb.2020.108701

Esteller, M., Corn, P. G., Baylin, S. B., & Herman, J. G. (2001). A gene hypermethylation profile of human cancer. *Cancer research*, *61*(8), 3225–3229.

Etienne-Manneville, S., & Hall, A. (2002). Rho GTPases in cell biology. *Nature*, *420*(6916), 629–635. https://doi.org/10.1038/nature01148

Ewald, A. J., Brenot, A., Duong, M., Chan, B. S., & Werb, Z. (2008). Collective epithelial migration and cell rearrangements drive mammary branching morphogenesis. *Developmental cell*, *14*(4), 570–581. https://doi.org/10.1016/j.devcel.2008.03.003

FARQUHAR, M. G., & PALADE, G. E. (1963). Junctional complexes in various epithelia. *The Journal of cell biology*, *17*(2), 375–412. https://doi.org/10.1083/jcb.17.2.375

Feinberg, A. P., & Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, *301*(5895), 89–92. https://doi.org/10.1038/301089a0

Fenaux, P., Mufti, G. J., Hellstrom-Lindberg, E., Santini, V., Finelli, C., Giagounidis, A., Schoch, R., Gattermann, N., Sanz, G., List, A., Gore, S. D., Seymour, J. F., Bennett, J. M., Byrd, J., Backstrom, J., Zimmerman, L., McKenzie, D., Beach, C., Silverman, L. R., & International Vidaza High-Risk MDS Survival Study Group (2009). Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. *The Lancet. Oncology*, *10*(3), 223–232. https://doi.org/10.1016/S1470-2045(09)70003-8

Ferrati, S., Gadok, A. K., Brunaugh, A. D., Zhao, C., Heersema, L. A., Smyth, H. D. C., & Stachowiak, J. C. (2017). Connexin membrane materials as potent inhibitors of breast cancer cell migration. *Journal of the Royal Society, Interface*, *14*(133), 20170313. https://doi.org/10.1098/rsif.2017.0313

Fiorini, C., Tilloy-Ellul, A., Chevalier, S., Charuel, C., & Pointis, G. (2004). Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. *Reproductive toxicology (Elmsford, N.Y.)*, *18*(3), 413–421. https://doi.org/10.1016/j.reprotox.2004.01.002

Fraga, M. F., Ballestar, E., Paz, M. F., Ropero, S., Setien, F., Ballestar, M. L., Heine-Suñer, D., Cigudosa, J. C., Urioste, M., Benitez, J., Boix-Chornet, M., Sanchez-Aguilera, A., Ling, C., Carlsson, E., Poulsen, P., Vaag, A., Stephan, Z., Spector, T. D., Wu, Y. Z., Plass, C., … Esteller, M. (2005). Epigenetic differences arise during the lifetime of monozygotic twins. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(30), 10604–10609. https://doi.org/10.1073/pnas.0500398102

Frantz, C., Stewart, K. M., & Weaver, V. M. (2010). The extracellular matrix at a glance. *Journal of cell science*, *123*(Pt 24), 4195–4200. https://doi.org/10.1242/jcs.023820

Friedenreich, C. M., Neilson, H. K., & Lynch, B. M. (2010). State of the epidemiological evidence on physical activity and cancer prevention. *European journal of cancer (Oxford, England : 1990)*, *46*(14), 2593–2604. https://doi.org/10.1016/j.ejca.2010.07.028

Furuse M. (2010). Molecular basis of the core structure of tight junctions. *Cold Spring Harbor perspectives in biology*, *2*(1), a002907. https://doi.org/10.1101/cshperspect.a002907

Gallicano, G. I., Kouklis, P., Bauer, C., Yin, M., Vasioukhin, V., Degenstein, L., & Fuchs, E. (1998). Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. *The Journal of cell biology*, *143*(7), 2009–2022. https://doi.org/10.1083/jcb.143.7.2009

Gandhi, K., Khan, S., Patrikar, M., Markad, A., Kumar, N., Choudhari, A., Sagar, P., & Indurkar, S. (2021). Exposure risk and environmental impacts of glyphosate: Highlights on the toxicity of herbicide co-formulants. *Environmental Challenges*, *4*, 100149. https://doi.org/10.1016/j.envc.2021.100149

Gao, Y., Widschwendter, M., & Teschendorff, A. E. (2018). DNA Methylation Patterns in Normal Tissue Correlate more Strongly with Breast Cancer Status than Copy-Number Variants. *EBioMedicine*, *31*, 243–252. https://doi.org/10.1016/j.ebiom.2018.04.025

Garrod, D., & Chidgey, M. (2008). Desmosome structure, composition and function. *Biochimica et biophysica acta*, *1778*(3), 572–587. https://doi.org/10.1016/j.bbamem.2007.07.014

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M. C., & Séralini, G. E. (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology*, *262*(3), 184–191. https://doi.org/10.1016/j.tox.2009.06.006

Giansanti, M. G., Gatti, M., & Bonaccorsi, S. (2001). The role of centrosomes and astral microtubules during asymmetric division of Drosophila neuroblasts. *Development (Cambridge, England)*, *128*(7), 1137–1145. https://doi.org/10.1242/dev.128.7.1137

Giepmans B. N. (2004). Gap junctions and connexin-interacting proteins. *Cardiovascular research*, *62*(2), 233–245. https://doi.org/10.1016/j.cardiores.2003.12.009

Giricz, O., Reynolds, P. A., Ramnauth, A., Liu, C., Wang, T., Stead, L., Childs, G., Rohan, T., Shapiro, N., Fineberg, S., Kenny, P. A., & Loudig, O. (2012). Hsa-miR-375 is differentially expressed during breast lobular neoplasia and promotes loss of mammary acinar polarity. *The Journal of pathology*, *226*(1), 108–119. https://doi.org/10.1002/path.2978

Godde, N. J., Sheridan, J. M., Smith, L. K., Pearson, H. B., Britt, K. L., Galea, R. C., Yates, L. L., Visvader, J. E., & Humbert, P. O. (2014). Scribble modulates the MAPK/Fra1 pathway to disrupt luminal and ductal integrity and suppress tumour formation in the mammary gland. *PLoS genetics*, *10*(5), e1004323. https://doi.org/10.1371/journal.pgen.1004323

Gong, Y., Yan, K., Lin, F., Anderson, K., Sotiriou, C., Andre, F., Holmes, F. A., Valero, V., Booser, D., Pippen, J. E., Jr, Vukelja, S., Gomez, H., Mejia, J., Barajas, L. J., Hess, K. R., Sneige, N., Hortobagyi, G. N., Pusztai, L., & Symmans, W. F. (2007). Determination of oestrogen-receptor status and ERBB2 status of breast carcinoma: a gene-expression profiling study. *The Lancet. Oncology*, *8*(3), 203–211. https://doi.org/10.1016/S1470-2045(07)70042-6

Gottardi, C. J., Wong, E., & Gumbiner, B. M. (2001). E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner. *The Journal of cell biology*, *153*(5), 1049–1060. https://doi.org/10.1083/jcb.153.5.1049

Gouon-Evans, V., Rothenberg, M. E., & Pollard, J. W. (2000). Postnatal mammary gland development requires macrophages and eosinophils. *Development (Cambridge, England)*, *127*(11), 2269–2282. https://doi.org/10.1242/dev.127.11.2269

Green, K. A., & Streuli, C. H. (2004). Apoptosis regulation in the mammary gland. *Cellular and molecular life sciences : CMLS*, *61*(15), 1867–1883. https://doi.org/10.1007/s00018-004-3366-y

Grifoni, D., Garoia, F., Schimanski, C. C., Schmitz, G., Laurenti, E., Galle, P. R., Pession, A., Cavicchi, S., & Strand, D. (2004). The human protein Hugl-1 substitutes for Drosophila lethal giant larvae tumour suppressor function in vivo. *Oncogene*, *23*(53), 8688–8694. https://doi.org/10.1038/sj.onc.1208023

Gudjonsson, T., Rønnov-Jessen, L., Villadsen, R., Rank, F., Bissell, M. J., & Petersen, O. W. (2002). Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. *Journal of cell science*, *115*(Pt 1), 39–50. https://doi.org/10.1242/jcs.115.1.39

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*(1), 57–70. https://doi.org/10.1016/s0092-8674(00)81683-9

Hatsell, S., Rowlands, T., Hiremath, M., et al. (2003). β-Catenin and Tcfs in Mammary Development and Cancer. *Journal of Mammary Gland Biology and Neoplasia, 8*, 145-158. https://doi.org/10.1023/A:1025834407156

Hervé, J. C., Derangeon, M., Bahbouhi, B., Mesnil, M., & Sarrouilhe, D. (2007). The connexin turnover, an important modulating factor of the level of cell-to-cell junctional communication: Comparison with other integral membrane proteins. *The Journal of Membrane Biology, 217*(1-3), 21-33. https://doi.org/10.1007/s00232-007-9048-2

Hiatt, R. A., & Brody, J. G. (2018). Environmental Determinants of Breast Cancer. *Annual review of public health*, *39*, 113–133. https://doi.org/10.1146/annurev-publhealth-040617-014101

Hirschi, K. K., Xu, C. E., Tsukamoto, T., & Sager, R. (1996). Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research*, *7*(7), 861–870.

Hoevel, T., Macek, R., Swisshelm, K., & Kubbies, M. (2004). Reexpression of the TJ protein CLDN1 induces apoptosis in breast tumor spheroids. *International journal of cancer*, *108*(3), 374–383. https://doi.org/10.1002/ijc.11571

Honeycutt, Z., & Rowlands, H. (2014). Glyphosate testing report: findings in American mothers' breast milk, urine and water. *Unpublished report, dated*, *7*.

Hoover, K. B., Liao, S. Y., & Bryant, P. J. (1998). Loss of the tight junction MAGUK ZO-1 in breast cancer: relationship to glandular differentiation and loss of heterozygosity. *The American journal of pathology*, *153*(6), 1767–1773. https://doi.org/10.1016/S0002-9440(10)65691-X

Houghton F. D. (2005). Role of gap junctions during early embryo development. *Reproduction (Cambridge, England)*, *129*(2), 129–135. https://doi.org/10.1530/rep.1.00277

Howard, B. A., & Gusterson, B. A. (2000). Human breast development. *Journal of mammary gland biology and neoplasia*, *5*(2), 119–137. https://doi.org/10.1023/a:1026487120779

Huang, G. Y., Wessels, A., Smith, B. R., Linask, K. K., Ewart, J. L., & Lo, C. W. (1998). Alteration in connexin 43 gap junction gene dosage impairs conotruncal heart development. *Developmental biology*, *198*(1), 32–44. https://doi.org/10.1006/dbio.1998.8891

Huang, P., Yang, J., Ning, J., Wang, M., & Song, Q. (2015). Atrazine Triggers DNA Damage Response and Induces DNA Double-Strand Breaks in MCF-10A Cells. *International Journal of Molecular Sciences*, *16*(12), 14353–14368. https://doi.org/10.3390/ijms160714353

Huettner, J. E., Lu, A., Qu, Y., Wu, Y., Kim, M., & McDonald, J. W. III (2006). Gap junctions and connexon hemichannels in human embryonic stem cells. *Stem Cells, 24,* 1654–1667.

Humphreys, R. C., & Hennighausen, L. (1999). Signal transducer and activator of transcription 5a influences mammary epithelial cell survival and tumorigenesis. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research*, *10*(10), 685–694.

Huszno, J., & Grzybowska, E. (2018). *TP53* mutations and SNPs as prognostic and predictive factors in patients with breast cancer. *Oncology letters*, *16*(1), 34–40. https://doi.org/10.3892/ol.2018.8627

Ikenouchi, J., Sasaki, H., Tsukita, S., Furuse, M., & Tsukita, S. (2008). Loss of occludin affects tricellular localization of tricellulin. *Molecular biology of the cell*, *19*(11), 4687–4693. https://doi.org/10.1091/mbc.e08-05-0530

Imbeault, S., Gauvin, L. G., Toeg, H. D., Pettit, A., Sorbara, C. D., Migahed, L., DesRoches, R., Menzies, A. S., Nishii, K., Paul, D. L., Simon, A. M., & Bennett, S. A. (2009). The extracellular matrix controls gap junction protein expression and function in postnatal hippocampal neural progenitor cells. *BMC neuroscience*, *10*, 13. https://doi.org/10.1186/1471-2202-10-13

Insua-Rodríguez, J., & Oskarsson, T. (2016). The extracellular matrix in breast cancer. *Advanced drug delivery reviews*, *97*, 41–55. https://doi.org/10.1016/j.addr.2015.12.017

Izdebska, M., Zielińska, W., Krajewski, A., Hałas-Wiśniewska, M., Mikołajczyk, K., Gagat, M., & Grzanka, A. (2021). Downregulation of MMP-9 Enhances the Anti-Migratory Effect of Cyclophosphamide in MDA-MB-231 and MCF-7 Breast Cancer Cell Lines. *International journal of molecular sciences*, *22*(23), 12783. https://doi.org/10.3390/ijms222312783

Jaffe, A. B., Kaji, N., Durgan, J., & Hall, A. (2008). Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis. *The Journal of cell biology*, *183*(4), 625–633. https://doi.org/10.1083/jcb.200807121

Jamieson, S., Going, J. J., D'Arcy, R., & George, W. D. (1998). Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. *The Journal of pathology*, *184*(1), 37–43. https://doi.org/10.1002/(SICI)1096-9896(199801)184:1<37::AID-PATH966>3.0.CO;2-D

Jayaseelan, V. P., Ramesh, A., & Arumugam, P. (2021). Breast cancer and DDT: putative interactions, associated gene alterations, and molecular pathways. *Environmental science and pollution research international*, *28*(21), 27162–27173. https://doi.org/10.1007/s11356-021-12489-6

Ji, H., Xu, L., Wang, Z., Fan, X., & Wu, L. (2018). Differential microRNA expression in the prefrontal cortex of mouse offspring induced by glyphosate exposure during pregnancy and lactation. *Experimental and therapeutic medicine*, *15*(3), 2457–2467. https://doi.org/10.3892/etm.2017.5669

Jiang, H., & Li, H. (2021). Prognostic values of tumoral MMP2 and MMP9 overexpression in breast cancer: a systematic review and meta-analysis. *BMC cancer*, *21*(1), 149. https://doi.org/10.1186/s12885-021-07860-2

Jin, Y., Feng, S. J., Qiu, S., Shao, N., & Zheng, J. H. (2017). LncRNA MALAT1 promotes proliferation and metastasis in epithelial ovarian cancer via the PI3K-AKT pathway. *European review for medical and pharmacological sciences*, *21*(14), 3176–3184.

Jirtle, R. L., & Skinner, M. K. (2007). Environmental epigenomics and disease susceptibility. *Nature reviews. Genetics*, *8*(4), 253–262. https://doi.org/10.1038/nrg2045

Jönsson, M., Borg, A., Nilbert, M., & Andersson, T. (2000). Involvement of adenomatous polyposis coli (APC)/beta-catenin signalling in human breast cancer. *European journal of cancer (Oxford, England : 1990)*, *36*(2), 242–248. https://doi.org/10.1016/s0959-8049(99)00276-2

Jönsson, M., Borg, A., Nilbert, M., & Andersson, T. (2000). Involvement of adenomatous polyposis coli (APC)/beta-catenin signalling in human breast cancer. *European journal of cancer (Oxford, England : 1990)*, *36*(2), 242–248. https://doi.org/10.1016/s0959-8049(99)00276-2

Joseph, C., Alsaleem, M., Orah, N. *et al.* Elevated MMP9 expression in breast cancer is a predictor of shorter patient survival. *Breast Cancer Res Treat* **182**, 267–282 (2020). https://doi.org/10.1007/s10549-020-05670-x

Kalra, J., Shao, Q., Qin, H., Thomas, T., Alaoui-Jamali, M. A., & Laird, D. W. (2006). Cx26 inhibits breast MDA-MB-435 cell tumorigenic properties by a gap junctional intercellular communication-independent mechanism. *Carcinogenesis*, *27*(12), 2528–2537. https://doi.org/10.1093/carcin/bgl110

Kamei, J., Toyofuku, T., & Hori, M. (2003). Negative regulation of p21 by beta-catenin/TCF signaling: a novel mechanism by which cell adhesion molecules regulate cell proliferation. *Biochemical and biophysical research communications*, *312*(2), 380–387. https://doi.org/10.1016/j.bbrc.2003.10.129

Kanczuga-Koda, L., Sulkowski, S., Lenczewski, A., Koda, M., Wincewicz, A., Baltaziak, M., & Sulkowska, M. (2006). Increased expression of connexins 26 and 43 in

lymph node metastases of breast cancer. *Journal of clinical pathology*, *59*(4), 429–433. https://doi.org/10.1136/jcp.2005.029272

Kaur, N., Swain, S. K., Banerjee, B. D., Sharma, T., & Krishnalata, T. (2019). Organochlorine pesticide exposure as a risk factor for breast cancer in young Indian women: A case-control study. *South Asian journal of cancer*, *8*(4), 212–214. https://doi.org/10.4103/sajc.sajc_427_18

Kechagioglou, P., Papi, R. M., Provatopoulou, X., Kalogera, E., Papadimitriou, E., Grigoropoulos, P., Nonni, A., Zografos, G., Kyriakidis, D. A., & Gounaris, A. (2014). Tumor suppressor PTEN in breast cancer: heterozygosity, mutations and protein expression. *Anticancer research*, *34*(3), 1387–1400.

Keely, P. J., Wu, J. E., & Santoro, S. A. (1995). The spatial and temporal expression of the alpha 2 beta 1 integrin and its ligands, collagen I, collagen IV, and laminin, suggest important roles in mouse mammary morphogenesis. *Differentiation; research in biological diversity*, *59*(1), 1–13. https://doi.org/10.1046/j.1432-0436.1995.5910001.x

Kerlikowske K. (2010). Epidemiology of ductal carcinoma in situ. *Journal of the National Cancer Institute. Monographs*, *2010*(41), 139–141. https://doi.org/10.1093/jncimonographs/lgq027

Khafaga, A. F., Mousa, S. A., Aleya, L., & Abdel-Daim, M. M. (2022). Three-dimensional (3D) cell culture: a valuable step in advancing treatments for human hepatocellular carcinoma. *Cancer cell international*, *22*(1), 243. https://doi.org/10.1186/s12935-022-02662-3

Khan, K., Hardy, R., Haq, A., Ogunbiyi, O., Morton, D., & Chidgey, M. (2006). Desmocollin switching in colorectal cancer. *British journal of cancer*, *95*(10), 1367–1370. https://doi.org/10.1038/sj.bjc.6603453

Kim, M., Datta, A., Brakeman, P., Yu, W., & Mostov, K. E. (2007). Polarity proteins PAR6 and aPKC regulate cell death through GSK-3beta in 3D epithelial morphogenesis. *Journal of cell science*, *120*(Pt 14), 2309–2317. https://doi.org/10.1242/jcs.007443

Kleinberg, D. L., Wood, T. L., Furth, P. A., & Lee, A. V. (2009). Growth hormone and insulin-like growth factor-I in the transition from normal mammary development to preneoplastic mammary lesions. *Endocrine reviews*, *30*(1), 51–74. https://doi.org/10.1210/er.2008-0022

Klingelhöfer, D., Braun, M., Brüggmann, D., & Groneberg, D. A. (2021). Glyphosate: How do ongoing controversies, market characteristics, and funding influence the global research landscape?. *The Science of the total environment*, *765*, 144271. https://doi.org/10.1016/j.scitotenv.2020.144271

Koledova Z. (2017). 3D Cell Culture: An Introduction. *Methods in molecular biology (Clifton, N.J.)*, *1612*, 1–11. https://doi.org/10.1007/978-1-4939-7021-6_1

Koledova, Z., & Lu, P. (2017). A 3D Fibroblast-Epithelium Co-culture Model for Understanding Microenvironmental Role in Branching Morphogenesis of the Mammary Gland. *Methods in molecular biology (Clifton, N.J.)*, *1501*, 217–231. https://doi.org/10.1007/978-1-4939-6475-8_10

Kominsky, S. L., Argani, P., Korz, D., Evron, E., Raman, V., Garrett, E., Rein, A., Sauter, G., Kallioniemi, O. P., & Sukumar, S. (2003). Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. *Oncogene*, *22*(13), 2021–2033. https://doi.org/10.1038/sj.onc.1206199

Krause, G., Winkler, L., Mueller, S. L., Haseloff, R. F., Piontek, J., & Blasig, I. E. (2008). Structure and function of claudins. *Biochimica et biophysica acta*, *1778*(3), 631–645. https://doi.org/10.1016/j.bbamem.2007.10.018

Krüger M, Schrödl W, Neuhaus J, Shehata A. Field Investigations of Glyphosate in Urine of Danish Dairy Cows. *J Environ Anal Toxicol*. 2013;03(05)

Schledorn, M.K. (2014). Detection of Glyphosate Residues in Animals and Humans. *Journal of Environmental and Analytical Toxicology, 4*, 1-5.

Kumar, N. M., & Gilula, N. B. (1996). The gap junction communication channel. *Cell*, *84*(3), 381–388. https://doi.org/10.1016/s0092-8674(00)81282-9

Kurebayashi, Y., Ojima, H., Tsujikawa, H., Kubota, N., Maehara, J., Abe, Y., Kitago, M., Shinoda, M., Kitagawa, Y., & Sakamoto, M. (2018). Landscape of immune microenvironment in hepatocellular carcinoma and its additional impact on histological and molecular classification. *Hepatology (Baltimore, Md.)*, *68*(3), 1025–1041. https://doi.org/10.1002/hep.29904

Kurzen, H., Münzing, I., & Hartschuh, W. (2003). Expression of desmosomal proteins in squamous cell carcinomas of the skin. *Journal of cutaneous pathology*, *30*(10), 621–630. https://doi.org/10.1034/j.1600-0560.2003.00122.x

Kwiatkowska, M., Reszka, E., Woźniak, K., Jabłońska, E., Michałowicz, J., & Bukowska, B. (2017). DNA damage and methylation induced by glyphosate in human peripheral blood mononuclear cells (in vitro study). *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, *105*, 93–98. https://doi.org/10.1016/j.fct.2017.03.051

Land, C. E., Tokunaga, M., Koyama, K., Soda, M., Preston, D. L., Nishimori, I., & Tokuoka, S. (2003). Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950-1990. *Radiation research*, *160*(6), 707–717. https://doi.org/10.1667/rr3082

Lanigan, F., McKiernan, E., Brennan, D. J., Hegarty, S., Millikan, R. C., McBryan, J., Jirstrom, K., Landberg, G., Martin, F., Duffy, M. J., & Gallagher, W. M. (2009). Increased claudin-4 expression is associated with poor prognosis and high tumour grade

in breast cancer. *International journal of cancer*, *124*(9), 2088–2097. https://doi.org/10.1002/ijc.24159

Lee, G. Y., Kenny, P. A., Lee, E. H., & Bissell, M. J. (2007). Three-dimensional culture models of normal and malignant breast epithelial cells. *Nature methods*, *4*(4), 359–365. https://doi.org/10.1038/nmeth1015

Lee, M., & Vasioukhin, V. (2008). Cell polarity and cancer--cell and tissue polarity as a non-canonical tumor suppressor. *Journal of cell science*, *121*(Pt 8), 1141–1150. https://doi.org/10.1242/jcs.016634

Lee, S. W., Tomasetto, C., Paul, D., Keyomarsi, K., & Sager, R. (1992). Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. *The Journal of cell biology*, *118*(5), 1213–1221. https://doi.org/10.1083/jcb.118.5.1213

Lelièvre S. A. (2009). Contributions of extracellular matrix signaling and tissue architecture to nuclear mechanisms and spatial organization of gene expression control. *Biochimica et biophysica acta*, *1790*(9), 925–935. https://doi.org/10.1016/j.bbagen.2009.03.013

Lelièvre, S. A., Weaver, V. M., Nickerson, J. A., Larabell, C. A., Bhaumik, A., Petersen, O. W., & Bissell, M. J. (1998). Tissue phenotype depends on reciprocal interactions between the extracellular matrix and the structural organization of the nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(25), 14711–14716. https://doi.org/10.1073/pnas.95.25.14711

Leroy, K., Haioun, C., Lepage, E., Le Métayer, N., Berger, F., Labouyrie, E., Meignin, V., Petit, B., Bastard, C., Salles, G., Gisselbrecht, C., Reyes, F., Gaulard, P., & Groupe d'Etude des Lymphomes de l'Adulte (2002). p53 gene mutations are associated with poor survival in low and low-intermediate risk diffuse large B-cell lymphomas. *Annals of oncology : official journal of the European Society for Medical Oncology*, *13*(7), 1108–1115. https://doi.org/10.1093/annonc/mdf185

Lesko, A. C., Goss, K. H., Yang, F. F., Schwertner, A., Hulur, I., Onel, K., & Prosperi, J. R. (2015). The APC tumor suppressor is required for epithelial cell polarization and three-dimensional morphogenesis. *Biochimica et biophysica acta*, *1853*(3), 711–723. https://doi.org/10.1016/j.bbamcr.2014.12.036

Li, F., Mao, G., Tong, D., Huang, J., Gu, L., Yang, W., & Li, G. M. (2013). The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutSα. *Cell*, *153*(3), 590–600. https://doi.org/10.1016/j.cell.2013.03.025

Li, G., Fridman, R., & Kim, H. R. (1999). Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. *Cancer research*, *59*(24), 6267–6275.

Li, P., Zhou, C., Yan, Y., Li, J., Liu, J., Zhang, Y., & Liu, P. (2019). Crumbs protein homolog 3 (CRB3) expression is associated with oestrogen and progesterone receptor

positivity in breast cancer. *Clinical and experimental pharmacology & physiology*, *46*(9), 837–844. https://doi.org/10.1111/1440-1681.13104

Li, Y., Wang, J., Wang, F., Gao, C., Cao, Y., & Wang, J. (2021). Identification of Specific Cell Subpopulations and Marker Genes in Ovarian Cancer Using Single-Cell RNA Sequencing. *BioMed research international*, *2021*, 1005793. https://doi.org/10.1155/2021/1005793

Lioni, M., Brafford, P., Andl, C., Rustgi, A., El-Deiry, W., Herlyn, M., & Smalley, K. S. (2007). Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells. *The American journal of pathology*, *170*(2), 709–721. https://doi.org/10.2353/ajpath.2007.060343

Locke, D., Perusinghe, N., Newman, T., Jayatilake, H., Evans, W. H., & Monaghan, P. (2000). Developmental expression and assembly of connexins into homomeric and heteromeric gap junction hemichannels in the mouse mammary gland. *Journal of cellular physiology*, *183*(2), 228–237. https://doi.org/10.1002/(SICI)1097-4652(200005)183:2<228::AID-JCP9>3.0.CO;2-Y

Longo, M., Zatterale, F., Naderi, J., Nigro, C., Oriente, F., Formisano, P., Miele, C., & Beguinot, F. (2020). Low-dose Bisphenol-A Promotes Epigenetic Changes at *Pparγ* Promoter in Adipose Precursor Cells. *Nutrients*, *12*(11), 3498. https://doi.org/10.3390/nu12113498

Lu, C., Han, H. D., Mangala, L. S., Ali-Fehmi, R., Newton, C. S., Ozbun, L., Armaiz-Pena, G. N., Hu, W., Stone, R. L., Munkarah, A., Ravoori, M. K., Shahzad, M. M., Lee, J. W., Mora, E., Langley, R. R., Carroll, A. R., Matsuo, K., Spannuth, W. A., Schmandt, R., Jennings, N. B., … Sood, A. K. (2010). Regulation of tumor angiogenesis by EZH2. *Cancer cell*, *18*(2), 185–197. https://doi.org/10.1016/j.ccr.2010.06.016

Lu, P., Weaver, V. M., & Werb, Z. (2012). The extracellular matrix: a dynamic niche in cancer progression. *The Journal of cell biology*, *196*(4), 395–406.

Lund, L. R., Rømer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Danø, K., & Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development (Cambridge, England)*, *122*(1), 181–193. https://doi.org/10.1242/dev.122.1.181

Ma, Y., Kanakousaki, K., & Buttitta, L. (2015). How the cell cycle impacts chromatin architecture and influences cell fate. *Frontiers in genetics*, *6*, 19. https://doi.org/10.3389/fgene.2015.00019

Ma, Y., Yu, L., Yan, W., Qiu, L., Zhang, J., & Jia, X. (2022). lncRNA GAS5 Sensitizes Breast Cancer Cells to Ionizing Radiation by Inhibiting DNA Repair. *BioMed Research International*, 2022, 1987519. https://doi.org/10.1155/2022/1987519

Macias, H., & Hinck, L. (2012). Mammary gland development. *Wiley interdisciplinary reviews. Developmental biology*, *1*(4), 533–557. https://doi.org/10.1002/wdev.35

Madhavan, D., Zucknick, M., Wallwiener, M., Cuk, K., Modugno, C., Scharpff, M., Schott, S., Heil, J., Turchinovich, A., Yang, R., Benner, A., Riethdorf, S., Trumpp, A., Sohn, C., Pantel, K., Schneeweiss, A., & Burwinkel, B. (2012). Circulating miRNAs as surrogate markers for circulating tumor cells and prognostic markers in metastatic breast cancer. *Clinical Cancer Research, 18*(21), 5972–5982. https://doi.org/10.1158/1078-0432.CCR-12-1402

Majidinia, M., & Yousefi, B. (2017). Breast tumor stroma: A driving force in the development of resistance to therapies. *Chemical Biology & Drug Design, 89*(3), 309-318. https://doi.org/10.1111/cbdd.12860

Mañas, F., Peralta, L., Raviolo, J., Ovando, H. G., Weyers, A., Ugnia, L., Cid, M. G., Larripa, I., & Gorla, N. (2009). Genotoxicity of glyphosate assessed by the comet assay and cytogenetic tests. *Environmental toxicology and pharmacology*, *28*(1), 37–41. https://doi.org/10.1016/j.etap.2009.02.001

Martin-Belmonte, F., & Mostov, K. (2008). Regulation of cell polarity during epithelial morphogenesis. *Current opinion in cell biology*, *20*(2), 227–234. https://doi.org/10.1016/j.ceb.2008.01.001

Martin, T. A., & Jiang, W. G. (2009). Loss of tight junction barrier function and its role in cancer metastasis. *Biochimica et Biophysica Acta, 1788*(4), 872–891. https://doi.org/10.1016/j.bbamem.2008.09.002

Martin, T. A., Watkins, G., Mansel, R. E., & Jiang, W. G. (2004). Loss of tight junction plaque molecules in breast cancer tissues is associated with a poor prognosis in patients with breast cancer. *European Journal of Cancer (Oxford, England: 1990), 40*(18), 2717–2725. https://doi.org/10.1016/j.ejca.2004.08.016

Martin, T. J., Gabure, S., Maise, J., Snipes, S., Peete, M., & Whalen, M. M. (2019). The organochlorine pesticides pentachlorophenol and dichlorodiphenyltrichloroethane increase secretion and production of interleukin 6 by human immune cells. *Environmental Toxicology and Pharmacology*, 72, 103263. https://doi.org/10.1016/j.etap.2019.103263

McLachlan, E., Shao, Q., Wang, H. L., Langlois, S., & Laird, D. W. (2006). Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis. *Cancer Research, 66*(20), 9886–9894. https://doi.org/10.1158/0008-5472.CAN-06-1726

Meeran, S. M., & Katiyar, S. K. (2008). Cell cycle control as a basis for cancer chemoprevention through dietary agents. *Frontiers in bioscience : a journal and virtual library*, *13*, 2191–2202. https://doi.org/10.2741/2834

Mehrgou, A., & Akouchekian, M. (2016). The importance of BRCA1 and BRCA2 genes mutations in breast cancer development. *Journal of Cellular Physiology, 231*(3), 574–585. https://doi.org/10.1002/jcp.25142

Mercadante, A. A., & Kasi, A. (2022). Genetics, Cancer Cell Cycle Phases. In *StatPearls*. StatPearls Publishing. Bookshelf ID: NBK563158

Mesnage, R., Benbrook, C., & Antoniou, M. N. (2019). Insight into the confusion over surfactant co-formulants in glyphosate-based herbicides. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, *128*, 137–145. https://doi.org/10.1016/j.fct.2019.03.053

Mesnage, R., Defarge, N., Spiroux de Vendômois, J., & Séralini, G. E. (2015). Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, *84*, 133–153.

Meyer, J. N., Leung, M. C., Rooney, J. P., Sendoel, A., Hengartner, M. O., Kisby, G. E., & Bess, A. S. (2013). Mitochondria as a target of environmental toxicants. *Toxicological sciences : an official journal of the Society of Toxicology*, *134*(1), 1–17. https://doi.org/10.1093/toxsci/kft102

Momiyama, M., Omori, Y., Ishizaki, Y., Nishikawa, Y., Tokairin, T., Ogawa, J., & Enomoto, K. (2003). Connexin26-mediated gap junctional communication reverses the malignant phenotype of MCF-7 breast cancer cells. *Cancer Science, 94*(6), 501–507. https://doi.org/10.1111/j.1349-7006.2003.tb01334.x

Monte, T. C., Garcia, J., Gentile, R., de Vasconcellos, M. C., Souza, J., Braga, B. V., & Maldonado, A., Jr (2016). In vivo and in vitro effects of the herbicide Roundup(®) on developmental stages of the trematode Echinostoma paraensei. *Experimental parasitology*, *169*, 43–50. https://doi.org/10.1016/j.exppara.2016.06.012

Morgan, G., & Wooding, F. B. (1982). A freeze-fracture study of tight junction structure in sheep mammary gland epithelium during pregnancy and lactation. *The Journal of Dairy Research, 49*(1), 1–11.

Muñoz, J. P., Araya-Osorio, R., Mera-Adasme, R., & Calaf, G. M. (2023). Glyphosate mimics 17β-estradiol effects promoting estrogen receptor alpha activity in breast cancer cells. *Chemosphere*, *313*, 137201. https://doi.org/10.1016/j.chemosphere.2022.137201

Naser Al Deen, N., AbouHaidar, M., & Talhouk, R. (2019). Connexin43 as a Tumor Suppressor: Proposed Connexin43 mRNA-circularRNAs-microRNAs Axis Towards Prevention and Early Detection in Breast Cancer. *Frontiers in medicine*, *6*, 192. https://doi.org/10.3389/fmed.2019.00192

Naser Al Deen, N., Atallah Lanman, N., Chittiboyina, S. *et al.* A risk progression breast epithelial 3D culture model reveals Cx43/hsa_circ_0077755/miR-182 as a biomarker axis for heightened risk of breast cancer initiation. *Sci Rep* **11**, 2626 (2021). https://doi.org/10.1038/s41598-021-82057-y

Nassar, F.J., Talhouk, R., Zgheib, N.K. *et al.* microRNA Expression in Ethnic Specific Early Stage Breast Cancer: an Integration and Comparative Analysis. *Sci Rep* **7**, 16829 (2017). https://doi.org/10.1038/s41598-017-16978-y

Naus, C. C., & Bani-Yaghoub, M. (1998). Gap junctional communication in the developing central nervous system. *Cell Biology International, 22*(11-12), 751–763. https://doi.org/10.1006/cbir.1998.0363

Naus, C. C., Bechberger, J. F., & Paul, D. L. (1991). Gap junction gene expression in human seizure disorder. *Experimental Neurology, 111*(2), 198–203. https://doi.org/10.1016/0014-4886(91)90018-P

Nguyen, D. A., Parlow, A. F., & Neville, M. C. (2001). Hormonal regulation of tight junction closure in the mouse mammary epithelium during the transition from pregnancy to lactation. *The Journal of Endocrinology, 170*(2), 347–356. https://doi.org/10.1677/joe.0.1700347

Nollet, F., Kools, P., & van Roy, F. (2000). Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *Journal of molecular biology, 299*(3), 551–572. https://doi.org/10.1006/jmbi.2000.3761

O'Brien, J., Martinson, H., Durand-Rougely, C., & Schedin, P. (2012). Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. *Development, 139*(2), 269-275. https://doi.org/10.1242/dev.071605

O'Brien, L. E., Jou, T. S., Pollack, A. L., Zhang, Q., Hansen, S. H., Yurchenco, P., & Mostov, K. E. (2001). Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nature cell biology, 3*(9), 831–838. https://doi.org/10.1038/ncb0901-831

O'Brien, L., Zegers, M. & Mostov, K. Building epithelial architecture: insights from three-dimensional culture models. *Nat Rev Mol Cell Biol* **3**, 531–537 (2002). https://doi.org/10.1038/nrm859

O'Connell, C. B., & Wang, Y. L. (2000). Mammalian spindle orientation and position respond to changes in cell shape in a dynein-dependent fashion. *Molecular Biology of the Cell*, 11, 1765-1774. https://doi.org/10.1091/mbc.11.5.1765

Ohashi, E., Murakumo, Y., Kanjo, N., Akagi, J., Masutani, C., Hanaoka, F., & Ohmori, H. (2004). Interaction of hREV1 with three human Y-family DNA polymerases. *Genes to cells : devoted to molecular & cellular mechanisms*, *9*(6), 523–531. https://doi.org/10.1111/j.1356-9597.2004.00747.x

Orman, A., Johnson, D. L., Comander, A., & Brockton, N. (2020). Breast Cancer: A Lifestyle Medicine Approach. *American journal of lifestyle medicine*, *14*(5), 483–494. https://doi.org/10.1177/1559827620913263

Osanai, M., Murata, M., Chiba, H., Kojima, T., & Sawada, N. (2007). Epigenetic silencing of claudin-6 promotes anchorage-independent growth of breast carcinoma cells. *Cancer science*, *98*(10), 1557–1562. https://doi.org/10.1111/j.1349-7006.2007.00569.x

Oshiro, M.M., Kim, C.J., Wozniak, R.J. *et al.* Epigenetic silencing of DSC3 is a common event in human breast cancer. *Breast Cancer Res* **7**, R669 (2005). https://doi.org/10.1186/bcr1273

Özdemir, S., Altun, S., & Arslan, H. (2018). Imidacloprid exposure cause the histopathological changes, activation of TNF-α, iNOS, 8-OHdG biomarkers, and alteration of caspase 3, iNOS, CYP1A, MT1 gene expression levels in common carp (Cyprinus carpio L.). *Toxicology Reports*, *5*, 125–133. https://doi.org/10.1016/j.toxrep.2017.12.019

Paplomata, E., & O'Regan, R. (2014). The PI3K/AKT/mTOR pathway in breast cancer: targets, trials and biomarkers. *Therapeutic advances in medical oncology*, *6*(4), 154–166. https://doi.org/10.1177/1758834014530023

Paszek, M. J., Zahir, N., Johnson, K. R., Lakins, J. N., Rozenberg, G. I., Gefen, A., Reinhart-King, C. A., Margulies, S. S., Dembo, M., Boettiger, D., Hammer, D. A., & Weaver, V. M. (2005). Tensional homeostasis and the malignant phenotype. *Cancer cell*, *8*(3), 241–254. https://doi.org/10.1016/j.ccr.2005.08.010

Patel, S., Alam, A., Pant, R., & Chattopadhyay, S. (2019). Wnt signaling and its significance within the tumor microenvironment: Novel therapeutic insights. *Frontiers in immunology*, *10*, 2872. https://doi.org/10.3389/fimmu.2019.02872

Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lønning, P. E., Børresen-Dale, A. L., Brown, P. O., & Botstein, D. (2000). Molecular portraits of human breast tumours. *Nature*, *406*(6797), 747–752. https://doi.org/10.1038/35021093

Petersen, O. W., Rønnov-Jessen, L., Howlett, A. R., & Bissell, M. J. (1992). Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(19), 9064–9068.

Pierce, D. A., & Preston, D. L. (2000). Radiation-related cancer risks at low doses among atomic bomb survivors. *Radiation research*, *154*(2), 178–186. https://doi.org/10.1667/0033-7587(2000)154[0178:rrcral]2.0.co;2

Piggin CL, Roden DL, Law AMK, Molloy MP, Krisp C, Swarbrick A, et al. (2020) ELF5 modulates the estrogen receptor cistrome in breast cancer. *PLoS Genet 16*(1): e1008531. https://doi.org/10.1371/journal.pgen.1008531

Plachot, C., & Lelièvre, S. A. (2004). DNA methylation control of tissue polarity and cellular differentiation in the mammary epithelium. *Experimental cell research*, *298*(1), 122–132. https://doi.org/10.1016/j.yexcr.2004.04.024

Plante, I., Stewart, M. K., Barr, K., Allan, A. L., & Laird, D. W. (2011). Cx43 suppresses mammary tumor metastasis to the lung in a Cx43 mutant mouse model of human disease. *Oncogene*, *30*(14), 1681–1692.

Pointis, G., Gilleron, J., Carette, D., & Segretain, D. (2010). Physiological and physiopathological aspects of connexins and communicating gap junctions in spermatogenesis. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *365*(1546), 1607–1620. https://doi.org/10.1098/rstb.2009.0114

Prasad, C. P., Mirza, S., Sharma, G., Prashad, R., DattaGupta, S., Rath, G., & Ralhan, R. (2008). Epigenetic alterations of CDH1 and APC genes: relationship with activation of Wnt/beta-catenin pathway in invasive ductal carcinoma of breast. *Life sciences*, *83*(9-10), 318–325. https://doi.org/10.1016/j.lfs.2008.06.019

Qi, M., & Xiong, X. (2018). Promoter hypermethylation of RARβ2, DAPK, hMLH1, p14, and p15 is associated with progression of breast cancer: A PRISMA-compliant meta-analysis. *Medicine, 97*(51), e13666. https://doi.org/10.1097/MD.0000000000013666

Qin, H., Shao, Q., Curtis, H., Galipeau, J., Belliveau, D. J., Wang, T., Alaoui-Jamali, M. A., & Laird, D. W. (2002). Retroviral delivery of connexin genes to human breast tumor cells inhibits in vivo tumor growth by a mechanism that is independent of significant gap junctional intercellular communication. *The Journal of biological chemistry, 277*(32), 29132–29138. https://doi.org/10.1074/jbc.M203592200

Rabih S. Talhouk, Antoine A. Khalil, Rachid Bajjani, Gilbert J. Rahme & Marwan E. El-Sabban. (2011). Gap junctions mediate STAT5-independent β-casein expression in CID-9 mammary epithelial cells. *Cell Communication & Adhesion, 18*(5), 104-116. https://doi.org/10.3109/15419061.2011.619926

Rao, A. K., Ziegler, Y. S., McLeod, I. X., Yates, J. R., & Nardulli, A. M. (2009). Thioredoxin and thioredoxin reductase influence estrogen receptor alpha-mediated gene expression in human breast cancer cells. *Journal of molecular endocrinology*, *43*(6), 251–261. https://doi.org/10.1677/JME-09-0053

Rawat, D., Bains, A., Chawla, P., Kaushik, R., Yadav, R., Kumar, A., Sridhar, K., & Sharma, M. (2023). Hazardous impacts of glyphosate on human and environment health: Occurrence and detection in food. *Chemosphere*, 138676. Advance online publication. https://doi.org/10.1016/j.chemosphere.2023.138676

Reynolds, P., Hurley, S., Goldberg, D. E., Anton-Culver, H., Bernstein, L., Deapen, D., Horn-Ross, P. L., Peel, D., Pinder, R., Ross, R. K., West, D., Wright, W. E., & Ziogas, A. (2004). Active Smoking, Household Passive Smoking, and Breast Cancer: Evidence

From the California Teachers Study. *JNCI: Journal of the National Cancer Institute, 96*(1), 29-37. https://doi.org/10.1093/jnci/djh005

Romano, M. A., Romano, R. M., Santos, L. D., Wisniewski, P., Campos, D. A., de Souza, P. B., Viau, P., Bernardi, M. M., Nunes, M. T., & de Oliveira, C. A. (2012). Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. *Archives of toxicology*, *86*(4), 663–673. https://doi.org/10.1007/s00204-011-0788-9

Ronckers, C. M., Erdmann, C. A., & Land, C. E. (2005). Radiation and breast cancer: a review of current evidence. Breast cancer research : *BCR, 7*(1), 21–32. https://doi.org/10.1186/bcr947

Roskelley, C. D., Desprez, P. Y., & Bissell, M. J. (1994). Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and bi-ochemical signal transduction. *Proceedings of the National Academy of Sciences of the United States of America, 91*(26), 12378–12382. https://doi.org/10.1073/pnas.91.26.12378

Roth, C., Rack, B., Müller, V., Janni, W., Pantel, K., & Schwarzenbach, H. (2010). Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer. *Breast cancer research : BCR*, *12*(6), R90. https://doi.org/10.1186/bcr2766

Runswick SK, O'Hare MJ, Jones L, Streuli CH, Garrod DR. (2001). Desmosomal adhesion regulates epithelial morphogenesis and cell positioning. *Nat Cell Biol, 3*(9), 823-830. https://doi.org/10.1038/ncb0901-823

Schedin, P., & Keely, P. J. (2011). Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression. *Cold Spring Harb Perspect Biol, 3*(1), a003228. https://doi.org/10.1101/cshperspect.a003228

Scholz, M., Nowak, P., Blaheta, R., Schuller, A., Menon, S., Cinatl, J., et al. (2004). Relocalization of endothelial cell β-catenin after coculture with activated neutrophils from patients undergoing cardiac surgery with cardiopulmonary bypass. *Journal of Investigative Surgery, 17*(3), 143-149.

Schönbrunn, E., Eschenburg, S., Shuttleworth, W. A., Schloss, J. V., Amrhein, N., Evans, J. N., & Kabsch, W. (2001). Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proc Natl Acad Sci U S A, 98*(4), 1376–1380. https://doi.org/10.1073/pnas.98.4.1376

Schwarzbauer, J. E., & DeSimone, D. W. (2011). Fibronectins, their fibrillogenesis, and in vivo functions. *Cold Spring Harb Perspect Biol, 3*(7), a005041. https://doi.org/10.1101/cshperspect.a005041

Schwarzenbach, H., Eichelser, C., Kropidlowski, J., Janni, W., Rack, B., & Pantel, K. (2012). Loss of heterozygosity at tumor suppressor genes detectable on fractionated circulating cell-free tumor DNA as indicator of breast cancer progression. *Clinical cancer research : an official journal of the American Association for Cancer Research*, *18*(20), 5719–5730. https://doi.org/10.1158/1078-0432.CCR-12-0142

Sengupta, S., Obiorah, I., Maximov, P. Y., Curpan, R., & Jordan, V. C. (2013). Molecular mechanism of action of bisphenol and bisphenol A mediated by oestrogen receptor alpha in growth and apoptosis of breast cancer cells. *British journal of pharmacology*, *169*(1), 167–178. https://doi.org/10.1111/bph.12122

Shackelford, R. E., Kaufmann, W. K., & Paules, R. S. (1999). Cell cycle control, checkpoint mechanisms, and genotoxic stress. *Environmental health perspectives*, *107 Suppl 1*(Suppl 1), 5–24. https://doi.org/10.1289/ehp.99107s15

Shamir, E. R., Pappalardo, E., Jorgens, D. M., Coutinho, K., Tsai, W. T., Aziz, K., Auer, M., Tran, P. T., Bader, J. S., & Ewald, A. J. (2014). Twist1-induced dissemination preserves epithelial identity and requires E-cadherin. *The Journal of cell biology*, *204*(5), 839–856. https://doi.org/10.1083/jcb.201306088

Shao, Q., Wang, H., McLachlan, E., Veitch, G. I., & Laird, D. W. (2005). Down-regulation of Cx43 by retroviral delivery of small interfering RNA promotes an aggressive breast cancer cell phenotype. *Cancer research*, *65*(7), 2705–2711. https://doi.org/10.1158/0008-5472.CAN-04-2367

Shen, H., Lan, Y., Zhao, Y. *et al.* The emerging roles of N6-methyladenosine RNA methylation in human cancers. *Biomark Res* **8**, 24 (2020). https://doi.org/10.1186/s40364-020-00203-6

Si, H., Chen, P., Li, H., & Wang, X. (2019). Long non-coding RNA H19 regulates cell growth and metastasis via miR-138 in breast cancer. *American journal of translational research*, *11*(5), 3213–3225.

Silverman, L. R., & Mufti, G. J. (2005). Methylation inhibitor therapy in the treatment of myelodysplastic syndrome. *Nature clinical practice. Oncology*, *2 Suppl 1*, S12–S23. https://doi.org/10.1038/ncponc0347

Söhl, G., & Willecke, K. (2004). Gap junctions and the connexin protein family. *Cardiovascular research*, *62*(2), 228–232. https://doi.org/10.1016/j.cardiores.2003.11.013

Song, C., Zhang, L., Wang, J. *et al.* High expression of microRNA-183/182/96 cluster as a prognostic biomarker for breast cancer. *Sci Rep* **6**, 24502 (2016). https://doi.org/10.1038/srep24502

Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Lønning, P. E., & Børresen-Dale, A. L. (2001). Gene expression

patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(19), 10869–10874. https://doi.org/10.1073/pnas.191367098

Soroceanu, L., Manning, T. J., Jr, & Sontheimer, H. (2001). Reduced expression of connexin-43 and functional gap junction coupling in human gliomas. *Glia*, *33*(2), 107–117. https://doi.org/10.1002/1098-1136

Soysal, S. D., Tzankov, A., & Muenst, S. E. (2015). Role of the Tumor Microenvironment in Breast Cancer. *Pathobiology : journal of immunopathology, molecular and cellular biology*, *82*(3-4), 142–152. https://doi.org/10.1159/000430499

Spagnol, G., Trease, A. J., Zheng, L., Gutierrez, M., Basu, I., Sarmiento, C., Moore, G., Cervantes, M., & Sorgen, P. L. (2018). Connexin43 Carboxyl-Terminal Domain Directly Interacts with β-Catenin. *International journal of molecular sciences*, *19*(6), 1562. https://doi.org/10.3390/ijms19061562

Sritana, N., Suriyo, T., Kanitwithayanun, J., Songvasin, B. H., Thiantanawat, A., & Satayavivad, J. (2018). Glyphosate induces growth of estrogen receptor alpha positive cholangiocarcinoma cells via non-genomic estrogen receptor/ERK1/2 signaling pathway. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, *118*, 595–607. https://doi.org/10.1016/j.fct.2018.06.014

Stadnyk A. W. (1994). Cytokine production by epithelial cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *8*(13), 1041–1047. https://doi.org/10.1096/fasebj.8.13.7926369

Stains, J. P., & Civitelli, R. (2005). Cell-to-cell interactions in bone. *Biochemical and biophysical research communications*, *328*(3), 721–727. https://doi.org/10.1016/j.bbrc.2004.11.078

Steinborn, A., Alder, L., Michalski, B., Zomer, P., Bendig, P., Martinez, S. A., Mol, H. G. J., Class, T. J., & Costa Pinheiro, N. (2016). Determination of Glyphosate Levels in Breast Milk Samples from Germany by LC-MS/MS and GC-MS/MS. *Journal of Agricultural and Food Chemistry*, *64*(6), 1414–1421. https://doi.org/10.1021/acs.jafc.5b05852

Stelwagen, K., Farr, V. C., Davis, S. R., & McFadden, H. A. (1998). Inhibition of milk secretion and the extent of filling of the bovine mammary gland. *Journal of Dairy Science*, 81, 376.

Stewart, M. K., Simek, J., & Laird, D. W. (2015). Insights into the role of connexins in mammary gland morphogenesis and function. *Reproduction (Cambridge, England)*, *149*(6), R279–R290. https://doi.org/10.1530/REP-14-0661

Stur, E., Aristizabal-Pachon, A. F., Peronni, K. C., Agostini, L. P., Waigel, S., Chariker, J., Miller, D. M., Thomas, S. D., Rezzoug, F., Detogni, R. S., Reis, R. S. D., Silva Junior, W. A., & Louro, I. D. (2019). Glyphosate-based herbicides at low doses affect canonical pathways in estrogen positive and negative breast cancer cell lines. *PloS one*, *14*(7), e0219610. https://doi.org/10.1371/journal.pone.0219610

Sultan, G., Zubair, S., Tayubi, I. A., Dahms, H. U., & Madar, I. H. (2019). Towards the early detection of ductal carcinoma (a common type of breast cancer) using biomarkers linked to the PPAR(γ) signaling pathway. *Bioinformation*, *15*(11), 799–805. https://doi.org/10.6026/97320630015799

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: a cancer journal for clinicians*, *71*(3), 209–249. https://doi.org/10.3322/caac.21660

Swain, S. M., Baselga, J., Kim, S. B., Ro, J., Semiglazov, V., Campone, M., Ciruelos, E., Ferrero, J. M., Schneeweiss, A., Heeson, S., Clark, E., Ross, G., Benyunes, M. C., Cortés, J., & CLEOPATRA Study Group (2015). Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. *The New England journal of medicine*, *372*(8), 724–734. https://doi.org/10.1056/NEJMoa1413513

Taddei, I., Deugnier, M. A., Faraldo, M. M., Petit, V., Bouvard, D., Medina, D., Fässler, R., Thiery, J. P., & Glukhova, M. A. (2008). Beta1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. *Nature cell biology*, *10*(6), 716–722. https://doi.org/10.1038/ncb1734

Takai, Y., Miyoshi, J., Ikeda, W., & Ogita, H. (2008). Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nature reviews. Molecular cell biology*, *9*(8), 603–615. https://doi.org/10.1038/nrm2457

Talhouk, R. S., Bissell, M. J., & Werb, Z. (1992). Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. *The Journal of cell biology*, *118*(5), 1271–1282. https://doi.org/10.1083/jcb.118.5.1271

Talhouk, R. S., Elble, R. C., Bassam, R., Daher, M., Sfeir, A., Mosleh, L. A., El-Khoury, H., Hamoui, S., Pauli, B. U., & El-Sabban, M. E. (2005). Developmental expression patterns and regulation of connexins in the mouse mammary gland: expression of connexin30 in lactogenesis. *Cell and tissue research*, *319*(1), 49–59. https://doi.org/10.1007/s00441-004-0915-5

Talhouk, R. S., Mroue, R., Mokalled, M., Abi-Mosleh, L., Nehme, R., Ismail, A., Khalil, A., Zaatari, M., & El-Sabban, M. E. (2008). Heterocellular interaction enhances recruitment of alpha and beta-catenins and ZO-2 into functional gap-junction complexes and induces gap junction-dependant differentiation of mammary epithelial cells. *Experimental cell research*, *314*(18), 3275–3291. https://doi.org/10.1016/j.yexcr.2008.07.030

Tarazona, J. V., Court-Marques, D., Tiramani, M., Reich, H., Pfeil, R., Istace, F., & Crivellente, F. (2017). Glyphosate toxicity and carcinogenicity: a review of the scientific basis of the European Union assessment and its differences with IARC. *Archives of toxicology*, *91*(8), 2723–2743. https://doi.org/10.1007/s00204-017-1962-5

Thomas, M., Laura, R., Hepner, K. *et al.* Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation. *Oncogene* **21**, 5088–5096 (2002). https://doi.org/10.1038/sj.onc.1205668

Thongprakaisang, S., Thiantanawat, A., Rangkadilok, N., Suriyo, T., & Satayavivad, J. (2013). Glyphosate induces human breast cancer cells growth via estrogen receptors. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, *59*, 129–136. https://doi.org/10.1016/j.fct.2013.05.057

Truffi, M., Sorrentino, L., & Corsi, F. (2020). Fibroblasts in the Tumor Microenvironment. *Advances in experimental medicine and biology*, *1234*, 15–29. https://doi.org/10.1007/978-3-030-37184-5_2

Tung, J., & Gilad, Y. (2013). Social environmental effects on gene regulation. *Cellular and molecular life sciences: CMLS*, *70*(22), 4323–4339. https://doi.org/10.1007/s00018-013-1357-6

Twigger, A. J., Engelbrecht, L. K., Bach, K., Schultz-Pernice, I., Pensa, S., Stenning, J., Petricca, S., Scheel, C. H., & Khaled, W. T. (2022). Transcriptional changes in the mammary gland during lactation revealed by single cell sequencing of cells from human milk. *Nature communications*, *13*(1), 562. https://doi.org/10.1038/s41467-021-27895-0

Utepbergenov, D. I., Fanning, A. S., & Anderson, J. M. (2006). Dimerization of the scaffolding protein ZO-1 through the second PDZ domain. *The Journal of biological chemistry*, *281*(34), 24671–24677. https://doi.org/10.1074/jbc.M512820200

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Jr, Lee, D. H., Shioda, T., Soto, A. M., vom Saal, F. S., Welshons, W. V., Zoeller, R. T., & Myers, J. P. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and non-monotonic dose responses. *Endocrine reviews*, *33*(3), 378–455. https://doi.org/10.1210/er.2011-1050

Van de Vijver, M. J., & Peterse, H. (2003). The diagnosis and management of pre-invasive breast disease: pathological diagnosis--problems with existing classifications. *Breast cancer research : BCR*, *5*(5), 269. https://doi.org/10.1186/bcr629

Van Keymeulen, A., Rocha, A. S., Ousset, M., Beck, B., Bouvencourt, G., Rock, J., Sharma, N., Dekoninck, S., & Blanpain, C. (2011). Distinct stem cells contribute to mammary gland development and maintenance. *Nature*, *479*(7372), 189–193. https://doi.org/10.1038/nature10573

Verderame, M., & Scudiero, R. (2019). How Glyphosate Impairs Liver Condition in the Field Lizard *Podarcis siculus* (Rafinesque-Schmaltz, 1810): Histological and Molecular Evidence. *BioMed research international*, *2019*, 4746283. https://doi.org/10.1155/2019/4746283

Vidi, P. A., Chandramouly, G., Gray, M., Wang, L., Liu, E., Kim, J. J., Roukos, V., Bissell, M. J., Moghe, P. V., & Lelièvre, S. A. (2012). Interconnected contribution of tissue morphogenesis and the nuclear protein NuMA to the DNA damage response. *Journal of cell science*, *125*(Pt 2), 350–361. https://doi.org/10.1242/jcs.089177

Wang, Q., & Margolis, B. (2007). Apical junctional complexes and cell polarity. *Kidney international*, *72*(12), 1448–1458. https://doi.org/10.1038/sj.ki.5002579

Wang, S., Seiwert, B., Kästner, M., Miltner, A., Schäffer, A., Reemtsma, T., Yang, Q., & Nowak, K. M. (2016). (Bio)degradation of glyphosate in water-sediment microcosms - A stable isotope co-labeling approach. *Water research*, *99*, 91–100. https://doi.org/10.1016/j.watres.2016.04.041

Watson C. J. (2006). Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. *Breast cancer research : BCR*, *8*(2), 203. https://doi.org/10.1186/bcr1401

Weir, M. L., Oppizzi, M. L., Henry, M. D., Onishi, A., Campbell, K. P., Bissell, M. J., & Muschler, J. L. (2006). Dystroglycan loss disrupts polarity and beta-casein induction in mammary epithelial cells by perturbing laminin anchoring. *Journal of cell science*, *119*(Pt 19), 4047–4058. https://doi.org/10.1242/jcs.03103

White, M. P., Elliott, L. R., Taylor, T., Wheeler, B. W., Spencer, A., Bone, A., Depledge, M. H., & Fleming, L. E. (2016). Recreational physical activity in natural environments and implications for health: A population based cross-sectional study in England. *Preventive medicine*, *91*, 383–388. https://doi.org/10.1016/j.ypmed.2016.08.023

Wiesen, J., & Werb, Z. (2000). Proteinases, cell cycle regulation, and apoptosis during mammary gland involution (minireview). *Molecular reproduction and development*, *56*(4), 534–540. https://doi.org/10.1002/1098-2795(200008)

Wijnhoven, B. P., Dinjens, W. N., & Pignatelli, M. (2000). E-cadherin-catenin cell-cell adhesion complex and human cancer. *The British journal of surgery*, *87*(8), 992–1005. https://doi.org/10.1046/j.1365-2168.2000.01513.x

Wilgenbus, K., Lentner, A., Kuckelkorn, R., Handt, S., & Mittermayer, C. (1992). Further evidence that acanthosis nigricans maligna is linked to enhanced secretion by the tumour of transforming growth factor alpha. *Archives of dermatological research*, *284*(5), 266–270. https://doi.org/10.1007/BF00372579

Williams, G. M., Aardema, M., Acquavella, J., Berry, S. C., Brusick, D., Burns, M. M., de Camargo, J. L. V., Garabrant, D., Greim, H. A., Kier, L. D., Kirkland, D. J., Marsh, G., Solomon, K. R., Sorahan, T., Roberts, A., & Weed, D. L. (2016). A review of the

carcinogenic potential of glyphosate by four independent expert panels and comparison to the IARC assessment. *Critical reviews in toxicology*, *46*(sup1), 3–20. https://doi.org/10.1080/10408444.2016.1214677

Witkiewicz, A. K., Knudsen, K. E., Dicker, A. P., & Knudsen, E. S. (2011). The meaning of p16(ink4a) expression in tumors: functional significance, clinical associations and future developments. *Cell cycle (Georgetown, Tex.)*, *10*(15), 2497–2503. https://doi.org/10.4161/cc.10.15.16776

Woodward, T. L., Mienaltowski, A. S., Modi, R. R., Bennett, J. M., & Haslam, S. Z. (2001). Fibronectin and the alpha(5)beta(1) integrin are under developmental and ovarian steroid regulation in the normal mouse mammary gland. *Endocrinology*, *142*(7), 3214–3222. https://doi.org/10.1210/endo.142.7.8273

Xu, R., Nelson, C. M., Muschler, J. L., Veiseh, M., Vonderhaar, B. K., & Bissell, M. J. (2009). Sustained activation of STAT5 is essential for chromatin remodeling and maintenance of mammary-specific function. *The Journal of cell biology*, *184*(1), 57–66. https://doi.org/10.1083/jcb.200807021

Xu, Y., & Vakoc, C. R. (2017). Targeting Cancer Cells with BET Bromodomain Inhibitors. *Cold Spring Harbor perspectives in medicine*, *7*(7), a026674. https://doi.org/10.1101/cshperspect.a026674

Xuan, R., Wang, J., Zhao, X., Li, Q., Wang, Y., Du, S., Duan, Q., Guo, Y., Ji, Z., & Chao, T. (2022). Transcriptome Analysis of Goat Mammary Gland Tissue Reveals the Adaptive Strategies and Molecular Mechanisms of Lactation and Involution. *International journal of molecular sciences*, *23*(22), 14424. https://doi.org/10.3390/ijms232214424

Yang, Y., Estrada, E. Y., Thompson, J. F., Liu, W., & Rosenberg, G. A. (2007). Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *27*(4), 697–709. https://doi.org/10.1038/sj.jcbfm.9600375

Yashiro, M., Nishioka, N., & Hirakawa, K. (2006). Decreased expression of the adhesion molecule desmoglein-2 is associated with diffuse-type gastric carcinoma. *European journal of cancer (Oxford, England : 1990)*, *42*(14), 2397–2403. https://doi.org/10.1016/j.ejca.2006.03.024

Yoshioka, N., Asano, M., Kuse, A., Mitsuhashi, T., Nagasaki, Y., & Ueno, Y. (2011). Rapid determination of glyphosate, glufosinate, bialaphos, and their major metabolites in serum by liquid chromatography-tandem mass spectrometry using hydrophilic interaction chromatography. *Journal of chromatography. A*, *1218*(23), 3675–3680. https://doi.org/10.1016/j.chroma.2011.04.021

Yu, W., Datta, A., Leroy, P., O'Brien, L. E., Mak, G., Jou, T. S., Matlin, K. S., Mostov, K. E., & Zegers, M. M. (2005). Beta1-integrin orients epithelial polarity via Rac1 and laminin. *Molecular biology of the cell*, *16*(2), 433–445. https://doi.org/10.1091/mbc.e04-05-0435

Zhang, L., Rana, I., Shaffer, R. M., Taioli, E., & Sheppard, L. (2019). Exposure to glyphosate-based herbicides and risk for non-Hodgkin lymphoma: A meta-analysis and supporting evidence. *Mutation research. Reviews in mutation research*, *781*, 186–206. https://doi.org/10.1016/j.mrrev.2019.02.001

Zhang, W., Wang, J., Song, J., Feng, Y., Zhang, S., Wang, N., Liu, S., Song, Z., Lian, K., & Kang, W. (2021). Effects of low-concentration glyphosate and aminomethyl phosphonic acid on zebrafish embryo development. *Ecotoxicology and environmental safety*, *226*, 112854. https://doi.org/10.1016/j.ecoenv.2021.112854

Zhang, Y. W., Nakayama, K., Nakayama, K., & Morita, I. (2003). A novel route for connexin 43 to inhibit cell proliferation: negative regulation of S-phase kinase-associated protein (Skp 2). *Cancer research*, *63*(7), 1623–1630.

Zhao, J., Pacenka, S., Wu, J., Richards, B. K., Steenhuis, T., Simpson, K., & Hay, A. G. (2018). Detection of glyphosate residues in companion animal feeds. *Environmental pollution (Barking, Essex : 1987)*, *243*(Pt B), 1113–1118. https://doi.org/10.1016/j.envpol.2018.08.100

Zhao, Y., Zheng, X., Zheng, Y., Chen, Y., Fei, W., Wang, F., & Zheng, C. (2021). Extracellular Matrix: Emerging Roles and Potential Therapeutic Targets for Breast Cancer. *Frontiers in oncology*, *11*, 650453. https://doi.org/10.3389/fonc.2021.650453

Zheng, Z., Zhu, H., Wan, Q., Liu, J., Xiao, Z., Siderovski, D. P., & Du, Q. (2010). LGN regulates mitotic spindle orientation during epithelial morphogenesis. *The Journal of cell biology*, *189*(2), 275–288. https://doi.org/10.1083/jcb.200910021

Zhou, W., Dinh, H.Q., Ramjan, Z. *et al.* DNA methylation loss in late-replicating domains is linked to mitotic cell division. *Nat Genet* **50**, 591–602 (2018). https://doi.org/10.1038/s41588-018-0073-4