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EFFECT OF ENDOTOXIN CHALLENGE ON NORMAL, TUMOR INITIATED, AND INVASIVE HUMAN BREAST CELLS

by
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AN ABSTRACT OF THE THESIS OF

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Breast cancer is the most common female cancer worldwide, recording high incidence rates caused by risk factors associated with urbanization and economic development. Inflammatory breast cancer (IBC) is the most lethal type of breast cancer, targeting young women, mainly after chronic inflammation. Interestingly, endotoxin (ET) is known to simulate inflammation-like conditions in several *in vitro* and *in vivo* models including mammary epithelial cells; however, little is known about the effect of ET-induced inflammation on breast cancer initiation events. In order to investigate the tumor initiating role of ET-induced inflammation, we monitored inflammatory mediators' response and cancer progression events of *in vitro* 2D cultures of normal mouse mammary epithelial cells (SCp2), 2D and 3D breast progression models of both, non-tumorigenic S1 cells, and intermediate stage of tumorigenesis S1-Connexin 43 knockouts (Cx43-KO S1), that can closely mimic the *in vivo* mammary epithelial morphology. Moderately invasive MCF-7 and highly invasive MDA-MB-231 human breast cancer cells were exploited in order to determine the effect of ET treatment on tumor invasion events.

Short-term treatment (48 hours) of SCp2 cells with ET concentrations ranging from 0.1 to 1 µg/ml upregulated the levels of MMP-9 produced by SCp2 cells in a dose-dependent manner. Long-term ET treatment (one month), but not short-term, enhanced the migration of SCp2 cells in wound healing assays. As for 2D cultures of the human S1 cells, 9-day ET treatment with concentrations ranging from 2 to 20 µg/ml upregulated the levels of MMP-9 in the conditioned media in a dose dependent manner. MMP-9 production by S1 cells was also upregulated upon long-term ET treatment (one month) with 10 µg/ml. Interestingly, the rate of S1 cells invasion though matrigel substrata was enhanced by both, 9-day and long-term treatment with 10 µg/ml ET. Moreover, immunofluorescent imaging of S1 acini in 3D cultures suggested lumen disruption and β-catenin re-localization in S1 acini subjected to long-term ET treatment with 10 µg/ml.

Tumor initiated Cx43-KO S1 cells showed a dose-dependent upregulation of MMP-9 levels upon 9-day treatment with increasing ET concentrations ranging between 2 and 10 µg/ml. Long-term treatment (one month) of Cx43-KO S1 cells with 10 µg/ml

also resulted in upregulation of MMP-9. Moreover, the invasion rate of Cx43-KO S1 through matrigel substrata was enhanced by both of the 9-day and long-term ET treatment.

Short-term ET treatment (48 hours) of human breast cancer cells with 0.1 and 1 μg/ml ET upregulated the levels of inflammatory mediators including nitric oxide (NO), interleukin 1-β (IL1-β), and MMPs in MDA-MB-231 conditioned media, in a dose-dependent manner. Moreover, long-term (one month) but not short-term ET treatment with 1 μg/ml enhanced the growth and migration rates of MCF-7 and MDA-MB-231 cells.

In conclusion, ET-induced inflammation enhanced inflammatory mediators' response and tumor progression events in normal, tumor-initiated and breast cancer cell lines. Our findings highlight the role of inflammatory insult in enhancing breast cancer initiation events, namely migration, invasion, and loss of normal differentiated morphology in normal breast cells. The results also suggest that such inflammatory insults can "add-injury" to already tumor-initiated or already invasive breast cells by increasing the rates of cell growth, migration and invasion.

CONTENTS

ILLUSTRATIONS

ABBREVIATIONS

%	Percent
/	Per
β-cat	β-catenin
µg	Micro Gram
µm	Micro Meter
2D	Two Dimensional
3D	Three Dimensional
AJs	Adherens Junctions
Akt	Protein kinase B (PKB)
ANOVA	Analysis of Variance
aPKC	Atypical Protein Kinase C
AU	Arbitrary Unit
bFGF	Basic Fibroblast Growth Factor
bp	Base Pair
BRCA1	Breast Cancer susceptibility gene 1
BRCA2	Breast Cancer susceptibility gene 2
Bves	Blood Vessel/Epicardial Substance
Ca	Calcium
CCL2	Chemokine (C-C motif) Ligand-2
CCN	(Cyr61, CTGF, Nov) Family
CDC42	Cell Division Control Protein 42
Cdc42	Cell division control protein 42 homolog
cDNA	Complimentary Deoxyribonucleic Acid

CID-9	Subpopulation of the Mouse Mammary Epithelial Cell Strain COMMA-1D
Cl	Chloride
CRB	Crumbs
CSC	Cancer Stem Cell
Cx	Connexin
Cx43	Connexin 43
Cx43-KO S1	Cx43-knockdown S1 cells
DAPI	4,6-diamino-2-phenylindole
DCIS	Ductal Carcinoma in Situ
Dlg	Discs Large Homologue
DMEM/F12	Dulbecco Modified Eagle Medium F12
DNA	Deoxyribonucleic Acid
Dsc	Desmocollin
Dsg	Desmoglein
E-cadherin	Epithelial Cadherin
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transtition
ER	Estrogen Receptor
ET	Endotoxin
et.al	et allii (and others)
FBS	Fetal Bovine Serum

fig	Figure
g	Acceleration of Gravity
G0	Gap 0
G1	Gap 1
G1	Gap 1
GAPDH	GlycerAldehyde 3-Phosphate DeHydrogenase
GJ	Gap Junction
GJA1	Gap Junction Alpha-1 Protein
GJIC	Gap Junction Intercellular Communication
GM	Growth Media
GPR30	G-protein Coupled Receptor 30
H1	Histone 1
hASC	Human Adipose Stem Cell
HCL	Hydrochloric Acid
HER2	Human Epidermal Growth Factor Receptor 2
HMECs	Human Mammary Epithelial Cells
HMT-3522 S1	Human Caucasian Breast Epithelial
hr	Hour
HTLV	Human T-Lymphotropic Virus
IBC	Inflammatory Breast Cancer
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IP3	1,4,5-inositol-trisphosphate
JAMs	Junctional Adhesion Molecules

LCIS	Lobular Carcinoma in Situ
LGL	Lethal Giant Larvae Homologue
LPS	Lipopolysaccharide
LSCM	Laser Scanning Fluorescent Confocal Microscope
M	Molar
MAPK	Mitogen-Activated Protein Kinase
MCF10A	Michigan Cancer Foundation cells number 10 A
MCF-7	Michigan Cancer Foundation cells number 7
MCP-1	Monocyte Chemotactic Protein-1
MD2	Myeloid Differential protein 2
MDA-MB-231	Monroe Dunaway Anderson Metastatic Breast cancer cells number 231
MDCK	Madin-Darby Canine Kidney cells
MET	Mesenchymal-Epithelial Transition
min	Minute
miRNA	MicroRNA
ml	milliLiter
mm	millimeter
MMP	Matrix Metalloproteinase
Na	Sodium
NA	Avogadro's Number
NaN3	Sodium Azide
N-cadherin	Neural Cadherin
NF-kB	Nuclear Factor-KappaB
NGF	Nerve Growth Factor

NIBC	Non-Inflammatory Breast Cancer
NO	Nitric Oxide
NuMA	Nuclear organizing Mitotic Apparatus
^{o}C	Degrees Celsius
p	Calculated Probability (p-value)
PALS1	Protein Associated with Lin-Seven 1
Par	Partitioning defective
PATJ	PALS1-Associated Tight Junction Protein
PBS	Phosphate Buffered Saline
P-cadherin	Pan-Cadherin
Pen-Strep	Penicillin-Streptomycin
PET/CT	Positron Emission Tomography/Computed Tomography
pg	Pico Gram
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PR	Progesterone Receptor
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
RhoC-GTPase	ras Homolog Family Member C-Guanosine Triphosphatase
RNA	RiboNucleicAcid
RPMI	Rose Park Memorial Institute
S1	Human Caucasian breast epithelial 3522 S1
SCRIB	Scribble Homologue
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean

shRNA	Small Hairpin RNA or Short Hairpin RNA
Slug	Human Embryonic Protein SNAI2
SNAI1	Zinc Finger Protein SNAI1 or Snail
SNAI2	Zinc Finger Protein SNAI2
Snail	Zinc Finger Protein SNAI1 or Snail
SOX2	SRY (Sex Determining Region Y)-Box 2
T	Time
TAM	Tumor Associated Macrophage
TGF-β	Transforming Growth Factor beta
TJ	Tight Junction
TLR	Toll-Like Receptor
TNBC	Triple Negative Breast Cancer
TNF-α	Tumor Necrosis Factor alpha
TNT	Tunneling Nanotube
TRI	Trizol
V	Volume
VEGF	Vascular Endothelial Growth Factor
WISP3	WNT1-Inducible- Signaling Pathway Protein 3
Wnt	Wingless-Related Integration Site
ZEB	Zinc Finger E-Box-Binding
ZO-1	Zonula Occluden-1

"Taught Man that which he knew not."

Quran [96:6]

A mes très chers parents…

CHAPTER I

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women and it constitutes the second leading cause of female cancer mortality after lung cancer (DeSantis et al. 2014). According to the American Cancer Society, one in eight women in the United States will experience breast cancer development throughout her lifetime (DeSantis et al. 2014). Likewise, the national cancer registry by the Lebanese Ministry of Public Health reported that breast cancer incidence is the most common among female cancers and contributes to one-third of the total cases in Lebanon; therefore, breast cancer is expected to be among the three most commonly diagnosed female cancers in 2018 (along with ovarian and non-Hodgkin cancers), which will account together for almost fifty percent of all cancers among women based on the projection statistical analysis (Shamseddine et al. 2014). Accumulated evidence has shown that family history is no more the sole explanation behind high percentages recorded for breast cancers with no genetic predisposition (Teegarden, Romieu, and Lelievre 2012); multiple factors may stand behind cancer occurrence. Thus, breast cancer incidence among females is on the rise, and although most of the efforts have been made to identify its risk factors, the molecular mechanisms/interactions that drive its initiation are not yet fully understood.

In order to decipher the early processes of breast cancer initiation, it is crucial to understand the aspects of the mammary gland proper functioning. An essential key feature in the mammary epithelium normal functioning and differentiation is the proper communication with its microenvironment. This involves the extracellular matrix

(ECM) and neighboring cells (Dbouk et al. 2009, Mroue, El-Sabban, and Talhouk 2011, El-Saghir et al. 2011). Equally compelling evidence demonstrates that the biochemical and physical nature of the stromal ECM microenvironment surrounding the epithelium and cell/cell interaction also contributes to breast homeostasis and tumorigenesis. Tissue architecture, as well, has a great impact in the regulation of normal and cancerous tissue behaviors of the mammary epithelium (Taraseviciute et al. 2010, Bazzoun, Lelievre, and Talhouk 2013). All this makes the mammary gland an excellent example of the inductive principle maintained in an adult organ.

Thus, one characteristic of the early stages of tumorigenesis in the mammary epithelium is the lack of the subsequent communication and intercellular adhesion. The formation of junction complexes at cell-cell contacts, better known as cell junctions, determines the architecture of the epithelium by mediating adhesion of cells to one another in an orderly manner, with tight junctions at the apex of cells (i.e., at the opposite cellular pole compared to the cellular pole in contact with the extracellular matrix) determining the limit between apical polarity and basolateral polarity. Cell junctions as gap junctions, composed of connexins and their associated proteins, also mediate communication by allowing the passage of signals and metabolites between neighboring cells. Tight junctions and gap junction proteins act in concert with epithelial cell polarity, defined as the asymmetrical distribution of cell junctions and polarity proteins (Zegers et al. 2003, Matter et al. 2005, Bazzoun, Lelievre, and Talhouk 2013) whereby the same proteins that control polarity dictate the allocation of cell junction proteins. In addition, both types of proteins, apical polarity proteins and cell junction proteins are classified as signaling hubs that regulate signal transduction

pathways involved in normal and cancer cell functions (Hansen and Bissell 2000, Bazzoun, Lelievre, and Talhouk 2013).

Therefore, research efforts to decipher the mechanisms through which disruption of cell junctions and polarity proteins contribute to the acquisition of tumor phenotype are paramount. Our lab is mostly interested in Connexin (Cx) transmembrane proteins, especially gap junction alpha-1 protein (GJA1), also known as connexin 43 (Cx43), for its important role in maintaining normal tissue homeostasis, controlling cell death, proliferation, and differentiation. This is done through facilitating gap junction intercellular communication (GJIC) between cells, as well as activating downstream signaling pathways (Dbouk et al. 2009). These features provide Cx43 with a tumor suppressive role in mammary glands, which has been proven in both *in vivo* and *in vitro* breast cancer experiments (Laird et al. 1999). We have recently suggested a regulatory role for Cx43 in apical polarity establishment that is a key property of epithelial tissues and is disrupted early on during tumorigenesis. HMT-3522 S1 non-neoplastic breast epithelial cells were used to decipher the mechanism through which Cx43 contributes to the homeostasis of the normal mammary epithelium (Submitted Bazzoun et al., 2016). Cx43-knockdown S1 cells (Cx43-KO S1) displayed phenotypic changes indicative of tumor initiation, including enhanced proliferation and cell cycle progression, along with mislocalization of membranous β-catenin, as demonstrated by its reduced apicolateral localization and increased basolateral distribution. In addition, the loss of Cx43 induced mislocalization of Scrib, one of the principal apicobasal polarity regulators, from apicolateral membrane domains, as well as mislocalization of ZO-1, apically-localized tight junction (Gianni et al.)-associated protein, indicating loss of apical polarity in Cx43-knockdown S1 cells (Submitted Bazzoun et al., 2016).

Interestingly, chronic inflammation is another factor that has been associated with malignant transformation in many tissues. Recently, pathways involved in inflammation and wound healing have been reported to enhance cancer stem cell (CSC) populations (Arnold et al. 2015). In this light, inflammatory breast cancer (IBC) was portrayed as the most lethal form of primary breast cancer targeting young women since it was coupled with a worse survival rate than other types of breast cancer. Clinically, IBC is defined by distinct features, including rapid onset within 6 months, erythema, edema of the breast, and a "peau d'orange" appearance to most areas of breast skin. Moreover, patients diagnosed with IBC manifested a positive metastatic lymph node involvement with distant metastasis at diagnosis. Studies identified specific biological markers that may be associated with IBC poor prognosis, and disease aggressiveness. For instance, IBC is characterized by amplification/over-expression of growth factor receptor HER2 and down regulation of hormone receptors ER/PR. Moreover, RhoC-GTPase is over-expressed in 90% of IBC tumors, which leads to up-regulation of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), and interleukin-8 (IL-8), contributing to a distinct type of angiogenic stroma formation in IBC carcinoma (Mohamed et al. 2014). Recent studies showed that EMT induced by inflammatory stimuli confers to cancer cells some mesenchymal stromal cell-like immune-modulatory properties, which could be a cue to decipher cancer progression and metastatic dissemination by favoring immune escape (Ricciardi et al. 2015). However, the association between chronic inflammation and transition of the normal breast epithelium into neoplastic tissue is not well understood.

To answer this question, we opt to study the role of inflammatory challenge in breast cancer initiation events in normal, tumor-initiated, and invasive human breast

cells. For this reason, an inflammation model has been proposed, in order to mimic the chronic inflammation events associated with malignant transformation. Previously, endotoxin (ET) was reported to simulate inflammation-like phenotype conditions in several *in vitro* and *in vivo* models. Epithelial cells (Haddad et al. 2001), umbilical vein endothelial cells (Pugin, Ulevitch, and Tobias 1993, Read et al. 1993), bovine and rodent mammary epithelial cells (Okada et al. 1997, Boudjellab et al. 1998, Safieh-Garabedian et al. 2004), and other cell types from different species were activated *in vitro* upon ET treatment. *In vivo*, inflammation and mastitis were induced in several animal models, when the mammary gland was exposed to ET (Colditz 1987, Oliver and Smith 1982, Shuster, Kehrli, and Stevens 1993). It was also shown that endotoxin activated NF-kB suppressed β-casein expression and upregulated gelatinases, cytokines, NGF and nitric oxide (NO) in rodent mammary cells (Safieh-Garabedian et al. 2004, Maalouf, Talhouk, and Schanbacher 2010).

Inflammation has been implicated in the initiation processes of several cancers (Grivennikov, Greten, and Karin 2010). The tumor microenvironment is important for the neoplastic initiation and invasion events, and interestingly, inflammatory microenvironment is involved in the malignant progression of breast cancer (Ham and Moon 2013). Recent studies have linked some inflammatory markers with breast cancer invasion such as matrix metallo-proteinases (MMPs). MMPs not only play an important role in the degradation of the ECM components upon cancer cell invasion and migration, but they also aid in recruiting multiple stromal cells during inflammation, including fibroblasts and leukocytes (Ham and Moon 2013). Consequently, stimulating inflammation in breast cells via ET treatment would constitute a valid approach to mimic the earlier events preceding the IBC onset. In this light, studying the

inflammatory biomarkers as well as the multiple inflammatory cellular mediators and their downstream effects due to a chronic insult is important for understanding cancer initiation {Reviewed by (Brenner et al. 2014)}.

Another important event in cancer invasion and metastasis is the epithelial-mesenchymal transition (EMT) (Huang, Chen, and Fang 2013). Although EMT has been long implicated in embryogenesis, it is now known that it is essential for providing cells with metastasis-initiation characteristics such as migration, invasion, and stem-like properties (Huang, Chen, and Fang 2013). These are due to the loss of polarity and cell-to-cell contact by epithelial cell layers, which then drastically remodel the cytoskeleton. Moreover, loss of the expression of proteins that promote cell-to-cell contact, such as E-cadherin and γ-catenin, may appear, and mesenchymal markers such as vimentin, fibronectin, N-cadherin, and the metalloproteinases MMP-2 and MMP-9 may be acquired by the cells, which enhance cell migration and invasion capabilities (Huang, Chen, and Fang 2013). The interactions between cancer cells and the tumor environment induce EMT through mechanisms such as the activation of several transcriptional repressors, mainly Snail, Slug and Twist. This is attained through various cellular signaling pathways such as NF-κB, Wnt and Hedgehog (Huang, Chen, and Fang 2013). After the cancer cells migrate to a compatible site, they undergo mesenchymal-epithelial transition (MET) to re-express E-cadherin and other epithelial markers (Huang, Chen, and Fang 2013).

In conclusion, little is known about the effect of ET-induced inflammation on breast cancer initiation events, and whether such inflammation would trigger loss of 3D morphological differentiation and apical polarity, displayed by lumen formation. Our study investigates the tumor initiating role of ET-induced inflammation, *in vitro*, on the

normal mammary epithelial model (SCp2) in addition to 2D and 3D breast progression models of both, non-tumorigenic normal S1 cells, and tumor initiated Cx43-KO S1 cells, that can closely mimic the *in vivo* mammary epithelial morphology. In addition, 2D cell cultures of the moderately invasive MCF-7 and the highly invasive MDA-MB-231 human breast cancer cells were also exploited in order to determine the effect of ET treatment on modulating tumor invasion and EMT associated events.

CHAPTER II

LITERATURE REVIEW

A. Breast Cancer Epidemiology and Types

1. Predominance and Mortality

Cancer, the second leading cause of death, has been recognized as a huge burden in economically developed countries as well as less industrialized nations (Torre et al. 2015) in terms of both epidemiology and cost (Valle, Tramalloni, and Bragazzi 2015). Despite the advancement in therapies, cancer incidence is still on the rise due to increased cancer risk in aging populations, and the adoption of lifestyles considered to be contributors to the increased prevalence of cancer risk factors. Those risk factors are typically associated with urbanization and economic development, and include smoking, overweight, poor diet, physical inactivity, and changing reproductive patterns (Torre et al. 2015). Malignancy of the breast, or breast cancer, is the most frequently diagnosed cancer among females (Torre et al. 2015) approximately accounting for one quarter of female cancers worldwide {Reviewed by (Makki 2015)} and ranking first among the causes of cancer related mortality in women (Bombonati and Sgroi 2011). Epidemiological studies estimate 40000 deaths per year of women in the United States due to this disease, this number is subject to fluctuation depending on several environmental and genetic risk factors (Jemal et al. 2010). Aside from effective breast cancer screening programs, maintaining a healthy body weight, minimizing alcohol intake and increasing physical activity, are considered the best available strategies to reduce the breast cancer development risk (Kushi et al. 2012). Current estimates, predict that one out of eight women would be diagnosed with breast cancer at some time during

her life. Breast cancer studies have clarified that family history alone cannot explain the huge percentage of breast cancers that develop with no genetic predisposition (Teegarden, Romieu, and Lelievre 2012). Despite slow improvement in prognosis due to the advent of early screening and marketing of targeted therapies, breast cancer mortality rate is still high mainly due to metastasis to other organs in the body rather than the primary tumor in the original cancerous mammary gland itself (Drasin, Robin, and Ford 2011, Scully et al. 2012).

2. Biomarkers and Subtypes

Breast cancer may target any of the mammary gland cells and it exhibits a wide span of morphological features, different immunohistochemical profiles, and unique histopathological subtypes that have specific clinical course and outcome. Although different types of breast sarcomas and lymphomas can be encountered, breast carcinomas were found to be the most common malignant lesion {Reviewed by (Makki 2015)}. This latter type constitutes more than 95% of breast malignancies (Vinay K 2010) and is usually classified primarily by its histological appearance, originating from the inner lining epithelium of the ducts or the lobules that supply the ducts with milk (Rosai 2011). Breast carcinomas are classified into two categories, non-invasive and invasive cancers, each of which is further divided into subtypes (DeSantis et al. 2014). Non-invasive breast cancers include Ductal Carcinoma in Situ (DCIS), also called stage zero, whereby abnormal cells are contained in the milk ducts of the breast and have not spread into the surrounding breast tissue. Moreover, non-invasive breast cancers include Lobular Carcinoma in Situ (LCIS) (Eheman et al. 2009), which is another subtype very similar to DCIS except that lesions appear most often in the terminal duct lobular units,

instead of being in the mammary ducts (Logan et al. 2015). Therefore, non-invasive breast cancer refers to the proliferation of neoplastic epithelial cells within the tubulo-lobular system of the breast, confined by the basement membrane and myo-epithelial cells of the ducts or lobules, without invading the stroma or blood and lymph or vessels (Pang, Gorringe, and Fox 2016). Conversely, invasive breast cancers consist of several subtypes where malignancy has spread from the original site, either the milk ducts or the lobules, into the surrounding breast tissue, lymph nodes and other parts of the body (Lakhani et al. 2012). Invasive ductal carcinoma is the most common subtype starting in the ducts of the breast, while invasive lobular carcinoma, the second most common type, originates in the lobules (Guiu et al. 2014). Less common types of invasive breast cancers with good prognosis comprise mucinous carcinoma, papillary carcinoma, and tubular carcinoma (Rakha et al. 2010). Moreover, the metaplastic breast cancer, inflammatory breast cancer (IBC) and Paget disease of the breast are other forms of breast cancer with a generally unfavorable outcome due to poor prognosis (Luini et al. 2007). Other histological special types of breast cancer include adenoid cystic, cribriform, medullary, apocrine, and lobular pleomorphic carcinomas (Dieci et al. 2014, Makki 2015). Interestingly, IBC is a rare, but aggressive form of breast cancer (Woodward and Cristofanilli 2009). Its main symptoms are swelling and redness of the breast. It accounts for about one to five percent of all breast cancers. With other forms of breast cancer, symptoms may not occur for years; however, with IBC, symptoms tend to occur within weeks or months (van Uden et al. 2015).

Based on histological observations of breast cancer tissues, breast cancer has been labeled as a heterogeneous disease (Holliday and Speirs 2011). Early in the last decade, many efforts have been concentrated in order to supplement the aforementioned

morphological classification of breast carcinoma with molecular parameters, in light of which, a better prediction of the tumor behavior could improve therapeutic strategies; consequently, breast cancers were classified into at least five different subtypes based on similarities in the gene expression profiles using the microarray technology: luminal A, luminal B, Human Epidermal growth factor Receptor 2 (HER2), basal, and normal (Perou et al. 2000). Luminal A and luminal B, being both Estrogen Receptor (ER) positive, are therefore amenable to hormone therapy, while the third subtype HER2 that is usually ER/PR negative, is still conquerable by HER2 targeted therapy as a result of HER2/neu over expression (Holliday and Speirs 2011). Luminal A has a good prognosis and is typically of low grade, while luminal B has a poorer prognosis (Rosai 2011). The HER2 overexpression group of breast cancer also implies a poor prognosis. It usually has a negative ER/PR while, as the name implies, a strong positive HER2/neu. These tumors are more likely to be of high grade and having lymph node metastasis {Reviewed by (Makki 2015)}. The basal group has a pattern of expression similar to basal epithelial cells and normal myoepithelial cells of mammary tissue. It is also called triple negative breast cancer (TNBC) due to the lack of expression of α-estrogen, progesterone, and HER2 receptors (Shawarby 2013). The vast majority of triple negative breast cancers are low-differentiated carcinomas, characterized by greater aggressiveness, frequent rate of local recurrence and organ metastases. Such cases are more common in younger women, and are associated with pathogenic mutations in the BRCA1 gene and in rare cases BRCA2, causing the occurrence of hereditary forms of breast cancer (Navratil et al. 2015). Therefore, chemotherapeutic treatments' main challenges are triple negative breast cancer subtypes such as the basal and normal phenotypes. Although they constitute around 15 percent of all breast cancers, TNBC

death rate is disproportionately higher than any other subtype (Kassam *et al.*, 2009). A sub-category of basal-like breast tumors have been identified by displaying low claudin3/4 and ki67 protein levels, thus termed the claudin-low subtype (Herschkowitz *et al.*, 2007). *In vitro* studies suggest that several cell lines are representative of this group, including MDA-MB-231 breast cancer cell lines, which are enriched with Epithelial Mesenchymal Transition (EMT) markers such as Vimentin and Twist (Neve et al. 2006).

B. Inflammation

1. Inflammatory Breast Cancer

As previously mentioned, among the different types of breast cancers, IBC represents a rare but aggressive form of cancer with negative prognosis and a high rate of recurrence (Diessner et al. 2015), comprising 1-5% of all breast cancers (Hance et al. 2005), and clinically characterized by a diffuse skin with an erysipeloid edge with no underlying mass (Sobin and Compton 2010). While primary inflammatory breast cancer refers to the development of breast carcinoma in a previously normal breast, secondary inflammatory breast carcinoma refers to the development of inflammatory skin changes associated with an invasive breast carcinoma that developed after a mastectomy of a non-inflammatory breast carcinoma (Kleer, van Golen, and Merajver 2000). Several conditions may be confused with IBC since they can mimic its clinical presentation, such as the case of nonpuerperal bacterial mastitis, leading to preventable delays in diagnosis and treatment (Peters, Kiesslich, and Pahnke 2002). *In vivo*, the dissemination of IBC carcinoma cells occurs through tumor embolism, a phenotype characterizing IBC carcinoma cells within lymphatic and blood vessels, whereby carcinoma cells

clump together retracting away from the surrounding endothelial lining of blood and lymphatic vessels (Gong 2008, Bonnier et al. 1995). This spread of IBC carcinoma cells in the form of clumps within blood and lymphatic vessels, results in distant metastasis in addition to multi-organ failure (Tsoi et al. 2010). IBC is accompanied by skin changes caused by tumor emboli within the dermal lymphatics, rather than being caused by infiltration of inflammatory cells. In terms of diagnosis, dermal lymphatic invasion without typical clinical findings is not sufficient for IBC diagnosis. Similarly, microscopical detection of tumor emboli in the dermal lymphatic vessels, although supportive of the diagnosis, is not required (Edge and Compton 2010). Among women with IBC, the median overall survival was less than 4 years, even with multimodality treatment options; however, in recent years, with the improvement of chemotherapeutical management, an increased survival has been noted (Anderson et al. 2005).

Despite the limited data regarding IBC risk factors, a high body mass index (BMI) has been positively associated with IBC diagnosis as compared to non-inflammatory breast cancer (NIBC) (Schairer et al. 2013, Levine and Veneroso 2008). Other risk factors associated with the diagnosis of IBC include younger age at first birth (Chang, Buzdar, and Hursting 1998) while an advanced age at first birth was associated with reduced risk of ER-negative IBC. Moreover, a reduced risk of both ER-positive and ER-negative IBC has been associated with higher level of high school and college education and correlated with a greater mammographic density among case subjects (Schairer et al. 2013). Diagnosis and staging of IBC have been tremendously improved with advances in imaging techniques such as ultrasonography, magnetic resonance imaging (MRI), and positron emission tomography/computed tomography (PET/CT)

(Groheux et al. 2013). The rapidly progressing IBC is accompanied by several symptoms including a painful tender, firm, and enlarged breast, a reddened, warm, and thickened skin edema resulting in a "peau d'orange appearance, thickening, erythema, and dermal lymphatic invasion (Lacerda et al. 2015). Hence, IBC mammography may show skin thickening over the breast, with or without a breast mass, a large area of calcification, and/or parenchymal distortion (Smoot et al. 2006). The fact that 30% of IBC patients present with a high rate of metastases requires an accurate initial staging in light of which an adequate systemic treatment can be planned (Yang et al. 2008). On the other hand, non-metastatic IBC treatment plan involves neoadjuvant chemotherapy, in addition to an ablative surgery in case a tumor-free resection margin is expected. Moreover, non-metastatic IBC treatment may involve local/regional radiotherapy as well as hormone therapy in case of HER2 or ER/PR positive tumors. This multimodal therapeutic approach, added to more targeted therapy becoming available, has significantly improved patient survival in recent years (Gianni et al. 2010, Hance et al. 2005). Nevertheless, IBC outcome is still poor, with a median disease-free survival of less than 2.5 years and an overall survival of 30–40% at 5 years (Kertmen et al. 2015, Yang and Cristofanilli 2006, Kleer, van Golen, and Merajver 2000).

2. Biomarkers of Inflammatory Breast Cancer

Several specific biological markers may be associated with IBC disease aggressiveness and poor prognosis. For instance, IBC is characterized by less hormone receptor expression compared to non-inflammatory breast cancer, mainly the estrogen and the progesterone receptors, and this has been consequently associated with a more aggressive clinical course and decreased survival rate (Zell et al. 2009, Cabioglu et al.

2007). Specifically, approximately 83% of IBC tumors lack estrogen receptor expression as compared to other forms of locally advanced breast cancers which are mostly ER positive (Woodward and Cristofanilli 2009). Despite a decreased estrogen receptor expression in IBC, estrogen signaling may be active in ER-negative IBC patients due to the presence of GPR30, a seven-transmembrane receptor belonging to the G-protein coupled receptor family, which was found in 69% of IBC patients, and is responsible of regulating cellular and physiological responsiveness to estrogen; therefore, it may be possible to exploit new potential therapies through non-classical estrogen-dependent pathways (Arias-Pulido et al. 2010).

The epidermal growth factor receptor family, involved in cell proliferation, survival, migration and differentiation consists of four members: epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2, 3 and 4 (HER2, HER3 and HER4) (Burgess 1987). EGFR was found to be overexpressed in 30% of IBC patients and associated with a significantly worse 5-year overall survival rate, as well as an increased risk of IBC recurrence compared to EGFR-negative IBC (Cabioglu et al. 2007). HER2 is a transmembrane receptor tyrosine kinase involved in signal transduction pathways leading to cell growth and differentiation (Ross et al. 2009). As HER2 is overexpressed, breast cancers become associated with further aggressiveness and higher recurrence rates as well as higher mortality. IBC patients were noted to have a high proportion of HER2-positive cases; however, HER2-positive status is not an independent adverse prognostic factor for survival among IBC patient cases (Zell et al. 2009, Dawood et al. 2008).

Moreover, loss of WNT1-inducible- signaling pathway protein 3 (WISP3) characterizes about 80% of IBC carcinoma tissue samples (van Golen et al. 1999).

WISP3, also known as CCN6, is a cysteine-rich protein playing a major role in inhibiting the invasive and angiogenic potential of IBC cells in both, tissue cultures as well as animal models (Kleer et al. 2002).

IBC emboli are also marked by over-expression of a number of genes including the ras homolog family member C-guanosine triphosphatase (RhoCGTPase), and E-cadherin (Gong 2008). The epithelial marker E-cadherin is a calcium dependent transmembrane glycoprotein mediating epithelial cell to cell adhesion (Kleer et al. 2001). E-cadherin is over expressed in IBC cells leading to an improved adherence of cells together, hence formation of tumor emboli. Studies have also suggested that E-cadherin, by promoting cell to cell contact, facilitates the dissemination of IBC within the lymphatic vessels, while maintaining the integrity of tumor emboli within dermal lymphatics (Gong 2008, Bonnier et al. 1995) The increased E-cadherin expression in IBC contributes to disease aggressiveness and a decreased survival rate (Kleer et al. 2001); however, in non-IBC, the loss of E-cadherin expression leads to increased tumor proliferation and metastasis associated with poor prognosis (Kowalski, Rubin, and Kleer 2003). On the other hand, 90% of IBC tumors show an overexpression of RhoC-GTPase playing an essential role in IBC metastatic behavior by increasing cellular motility and invasion, cytoskeletal assembly, and cell adhesion. RhoC-GTPase also controls aspects of the cytoskeletal reorganization by inducing focal adhesion contacts formation and actin stress fiber (van Golen et al. 2002, van Golen et al. 2000, Van den Eynden et al. 2004).

Other distinctive features of IBC were detected by DNA microarrays studies which showed an over-expression of Toll-like receptors (TLR) in IBC tissues versus non-IBC tissues (Yamauchi et al. 2012). Over-expression of TLR, usually expressed in

response to microbial or viral infections, suggests infiltration of IBC by immune cells and possibility of viral etiology in IBC progression (Van Laere et al. 2013).

Cues from the inflammatory cells affect IBC dissemination within the tumor microenvironment. For instance, macrophages are known to be the major inflammatory cells able to infiltrate breast and other various types of tumors (Pollard 2008, Mukhtar et al. 2011), contributing to high levels of growth factors, cytokines and hormones, in the tumor microenvironment (Aaltomaa et al. 1992, Georgiannos et al. 2003). Tumor associated macrophages (TAM) available within the tumor microenvironment are differentiated into heterogeneous subpopulations, including "classically activated macrophages" and "regulatory macrophages". Classically activated macrophages secrete pro-inflammatory and inflammatory mediators and recruit T-cells as in an early inflammatory response (Ojalvo et al. 2009), while regulatory macrophages express anti-inflammatory cytokines and increase tumor growth, invasion, and metastasis (Mosser and Edwards 2008). Since macrophages secrete soluble mediators inducing migration, invasion, and metastasis (Condeelis and Pollard 2006, Mantovani et al. 2006), breast TAM have been strongly associated with poor prognosis (Lewis et al. 1995, Leek et al. 1996). Among the soluble mediators secreted by TAM are the matrix metalloproteinases- 2 and 9 (MMP-2 and MMP-9), enzymes capable of degrading the basement membrane components, subsequently facilitating tumor cell motility and dissemination (Mantovani et al. 2006, Hagemann et al. 2005)

Cancer cells secrete proteases and cytokines that facilitate extracellular matrix degradation, invasion and motility. Proteases, such as cysteine cathepsins, enable them to invade and metastasize via degrading basement membranes and extracellular matrix proteins (Mohamed and Sloane 2006a). Proteases also modulate the secretion and

activity of cytokines which in turn influence the invasive and metastatic behavior of cancer cells (Opdenakker and Van Damme 1992). Proteases in non-IBC are well studied and their roles well investigated; however, when it comes to IBC, they are poorly studied despite their great contribution in conferring specific invasiveness properties to IBC carcinoma cells such as degradation of extracellular matrix, cell motility, and metastasis (Van den Eynden et al. 2006). Moreover, cell surface proteins namely caveolin-1 and -2, found on the surface lipid raft caveolae have been also linked to IBC by serving as a home for inactive proteases namely the inactive precursors of cysteine cathepsins secreted from both transformed and tumour cells which associate with binding partners on the cell surface prior to their activation by peritumoral acidification and tumor–stroma interactions, leading to pericellular proteolysis adjacent to tumor cells or {Reviewed (Mohamed and Sloane 2006b)}.

Cytokines and chemokines within the tumor microenvironment modulate the cross talk between cells; however, their role in IBC is not well understood (Mohamed et al. 2014). Few studies revealed the role of cytokines in some IBC cell lines. For instance, the canine inflammatory mammary cancer model, is characterized by higher serum levels of interleukins (IL) namely IL-6, IL-8, and IL-10 as compared to thecanine non-inflammatory malignant mammary cancer (de Andres et al. 2013). Two other IBC cell lines, SUM149 and SUM190, similarly secrete IL-6 and IL-8 cytokines contributing to an increase in the self-renewal potential of stem cells via the Notch signaling pathway (Debeb et al. 2012). Moreover, when measuring the level of cytokines in IBC patients, IL-6 was found to be overexpressed (Bieche et al. 2004) and recorded significantly higher levels compared to non-IBC patients (Drygin et al. 2011). Other major cytokines and chemokines have been portrayed as role players in IBC. While IL-

1 is a potential inducer of IL-8 production by breast cancer cells *in vitro* (Pantschenko et al. 2003), IL-1 β is involved in breast cancer progression and relapse (Miller et al. 2000, Pantschenko et al. 2003). IL-8 is an angiogenic stimulator (Lin et al. 2004) and is capable of promoting IBC carcinoma cells invasion and motility through induction of PI3 k/Akt signaling pathway (Mohamed 2012). TNF-α is another contributor to the epithelial mesenchymal transition (EMT) in breast cancer cells (Li et al. 2012, Wu et al. 2011), in addition to its role as a mediator for production of IL-6 and IL-8 (Debeb et al. 2012). The monocyte chemotactic protein-1 (MCP-1) also known as chemokine (C-C motif) ligand-2 (CCL2) interferes by promoting breast cancer growth and metastasis (Qian et al. 2011), CCL2 and CCL5 being upregulated by TNF-α and IL-1β (Raman et al. 2007).

C. Tissue Architecture and Cancer

Equally compelling evidence demonstrates that the biochemical and physical nature of the stromal extracellular matrix (ECM) microenvironment surrounding the epithelium and cell/cell interaction also contributes to breast homeostasis and tumorigenesis. Tissue architecture, as well, has a great impact in the regulation of normal and cancerous tissue behaviors of the mammary epithelium (Taraseviciute et al. 2010, Bazzoun, Lelievre, and Talhouk 2013). All this makes the mammary gland an excellent example of the inductive principle maintained in an adult organ. In other words, tissue composition and the state of differentiation change drastically according to functional requirement and are dependent on mesenchymal-parenchymal interactions, extracellular matrix, and local paracrine and endocrine interactive stimuli (Munarini et

al. 2002). Interestingly, when such interactions are altered they might lead to tumor development (Hansen and Bissell 2000, Lelievre 2009, Talhouk 2012).

Aside from the aforementioned microenvironement requirement, epithelial differentiation and function depend on three major aspects: establishment of intercellular junctions and adhesion, basoapical polarity and proper mitotic spindle orientation (Dickinson, Nelson, and Weis 2011). Cellular differentiation is characterized by a cell acquiring a defined structure that enables it to interact with other cells and with its microenvironment, including the stroma, hormones, growth factors and the extracellular matrix, to perform specific functions, as opposed to a stem cell that has no defined "identity" and can give rise to various cell types. To attain a wide range of functions such as protection, secretion and absorption, epithelial cells form highly organized tissues characterized by the tight regulation of polarity and cell-cell junction complexes. Epithelial cell polarity is defined as the asymmetrical distribution of cell junction and polarity proteins (O'Brien, Zegers, and Mostov 2002, Matter et al. 2005, Martin-Belmonte and Mostov 2008, Bazzoun, Lelievre, and Talhouk 2013).

1. Cell Junctions in Breast Carcinogenesis

Importantly, every cell has the capacity of sensing its directly contacting neighbors. This is why cell junctions, represented by junction complexes at cell-to-cell contacts, not only mediate adhesion between a cell and another, but also ensure a systematic well-ordered communication with the cellular neighborhood. The complex proteins assembling at the intercellular junctions are now perceived as key contributors to signal transduction, rather than physical channels or scaffolds governing cell contact and tissue architecture. Consequently, cell adhesions contribute to a harmonized well-

coordinated cell behavior, which determines the normal architecture of the epithelium. 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). Gap junctions for instance are responsible for mammary epithelial cell differentiation, and the disruption of these protein complexes contributes to the loss of the differentiated phenotype (Talhouk et al. 2008).

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). This morphological feature of TJs is tailored to fit their function whereby they may be referred to as "permeability seals" that restrict the leak of solutes through the intercellular space, specifically, they restrict lipid diffusion between apical and basolateral domains, and control solute diffusion based on size and charge (Balda and Matter 2008, Anderson and Van Itallie 2009). Since TJs are localized at the apex of cells, they mark the apical side and are major contributors to tissue polarity. They are also considered key players in determining the limit between apical and basolateral sides of the membranes. Although purification of TJs is difficult, some of their molecular components have been identified. TJs associated proteins comprise transmembrane claudins, occludins, tricellulin, Zona Occludens (ZO) family members, junctional adhesion molecules (JAMs), CRB-3, and blood vessel/epicardial substance (Bves) (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). The extracellular domains of the latter are in control of the diffusion of small hydrophilic molecules. The

first TJ protein identified was ZO-1 which is a scaffold protein of the TJ cytoplasmic plaque. Moreover, ZO-1 is suggested to be a common binding partner of TJs, AJs and GJs proteins (Utepbergenov, Fanning, and Anderson 2006, Giepmans 2004). During cancer metastasis or normal tissue development, occludins induced TJ disassembly through regulation of TGF-β type I receptor localization. This disassembly is key feature of epithelial-to-mesenchymal (EMT) transition, a phenomenon during which cells acquire mesenchymal features enabling them to detach from the epithelium (Barrios-Rodiles et al. 2005). Moreover, cancer progression has been linked to TJ proteins modulation whereby breast cancer cell lines demonstrated decreased expression levels of occludins and claudin-1, 4 and 6 (Osanai et al. 2007, Hoevel et al. 2004). Interestingly, TJ proteins claudin and occludin, along with their associated proteins work hand in hand with the apical polarity regulators including Crb and Par complexes, Scrib, Dlg, and Lgl proteins, in order to modulate signal transduction. Therefore, the role of tight junctions is no longer limited to sealing the intercellular space, but goes into functioning as signaling hubs capable of modulating the distribution of transcription regulators between nuclear compartment and tight junction complexes, in addition to modulating chromatin-associated complexes {Reviewed by (Lelievre 2009)}.

On the other hand, adherens junctions facilitate intercellular adhesion and include the classic cadherins and nectins. Classical cadherins such as E-cadherin, N-cadherin and P-cadherin anchor cytoskeletal intermediate filaments to the membrane and permit strong cellular adhesion (Lanigan et al. 2009). E-cadherin is implicated in cell proliferation, differentiation, gene transcription and cell adhesion by negatively regulating the Wnt signaling pathway. For instance, β-catenin binds to E-cadherin, and

is thus recruited to the cell membrane away from the nucleus, consequently preventing its pro-proliferative potential (Wijnhoven, Dinjens, and Pignatelli 2000). Interestingly, blocking E-cadherin in the mammary ducts inhibits the growth of the epithelial tissue by interfering with downstream signaling pathways initiated at the AJ complex (Lanigan et al. 2009). Cancer alters cadherins levels whereby breast cancer progression correlates with a down regulation of E-cadherin (Cowin, Rowlands, and Hatsell 2005). Paradoxically, the expression of E-cadherin increases uniquely in IBC cell lines but not in non-IBC cell lines (Cohen et al. 2015). As previously stated, tumor emboli express cell adhesion molecules responsible of maintaining the tumor cells together; and 90 % of human IBCs are associated with increased E-cadherin which indicates that the gain of E-cadherin axis contributes to the IBC phenotype (Fernandez et al. 2013).

As for desmosomal junctions, they enclose transmembrane proteins from the desmocollin and desmoglein subfamilies, in addition to cadherins (Garrod and Chidgey 2008). These junctions maintain cell-to-cell adhesion, and are involved in other cellular processes due to the fact that they are dynamic structures that serve as signaling hubs. For instance, they can alter the differentiation and epidermal hyperproliferation in transgenic mice through the differential expression of desmoglein 1 (Dsg1) and desmocollin 1 (Dsc1) in the suprabasal layers of epidermis, versus Dsg3 and Dsc3 which are more expressed basally. This differential expression forms an adhesive gradient responsible of communicating positional information, hence contributing to epidermal morphogenesis and altering the proliferation and differentiation of keratinocytes (Merritt et al. 2002). Moreover, breast cancer metastasis is marked by a down regulation of desmoplakin correlating with increased cell proliferation and tumor size (Knudsen and Wheelock 2005). On the other hand, engineered transgenic mice

with altered distribution of desmoglein 3 in the epidermis as found in normal oral mucous membranes, died shortly after birth with severe dehydration reflecting excessive trans-epidermal water loss. This suggests that differential expression of desmoglein isoforms alters the epidermal permeability barrier function which is a major function of the epidermis (Elias et al. 2001). Furthermore, desmosomal adhesion could be affected to a great extent by estrogen levels, whereby estrogens can upregulate four desmosomal proteins: desmocollin, γ-catenin, plakophilin and desmoplakin, therefore increasing the formation of desmosomes in normal and malignant breast cells. This estrogen modulation of desmosome formation makes this junction a subject for further investigation in mammary gland differentiation and cancer development (Maynadier et al. 2012).

Gap junctions are structures that provide direct pathways of communication. They are composed of connexins and their associated proteins. Located right beneath the TJs, GJs allow for direct exchange of signaling molecules and metabolites between neighboring cells and enable several other functions, such as rapid transmission of action potentials and diffusion of metabolites, nutrients, secondary messengers below 1200 Da in size such as 1,4,5-inositol-trisphosphate (IP3), calcium ions, and cyclic nucleotides, hence participating in the modulation of gene transcription, proliferation and apoptosis (Guttman and Finlay 2009). Every six transmembrane proteins called connexins (Cxs) assemble to form one connexon, and when two connexons pair together, a GJ channel is formed. Cxs are not only perceived as routes for channeling metabolites, but also as signaling nodes affecting both normal and cancer cell functions {Reviewed by (Bazzoun, Lelievre, and Talhouk 2013)}. Murine mammary glands have been shown to house functional GJs (Talhouk et al. 2005, Talhouk et al. 2008), and the

spatial and temporal expression of Cxs and their extensive contribution to the mammary gland development have been extensively studied {Reviewed by (El-Sabban et al. 2003, McLachlan, Shao, and Laird 2007). Interestingly, major cellular mechanisms, such as migration, are directed by polarity and cell-cell junction proteins interactions whether in normal or cancerous contexts. Proteins that control polarity are capable of dictating the distribution of cell junction proteins, hence acting in concert. They are further classified as signaling hubs that regulate signal transduction pathways involved in normal and cancer cell functions {Reviewed by (Bazzoun, Lelievre, and Talhouk 2013)}. The crosstalk between gap junctions and polarity is not to be denied. A direct interaction between connexins and polarity proteins has been described through Drebrin, the "developmentally regulated brain protein" (Butkevich et al. 2004). Consequently, Cxs are no longer perceived solely as structural proteins, but rather as interactive signaling hubs exerting coordinated interactions with TJ, AJ and polarity proteins throughout the epithelial differentiation.

Tunneling nanotubes, similar to GJs, facilitate intracellular transfer of molecules; however, TNTs mediate the exchange of small molecules, vesicles and organelles, as contrasted to GJs. Studies have also revealed that, in presence of functional gap junctions, TNTs are capable of electrical coupling whereby depolarization signals are transferred between cells for distances of up to at least 70 μm (Wang et al. 2010). TNTs were also shown to contribute to mechano-transduction by transferring a mechanical signal between pairs of TNT coupled cells (Orr et al. 2006). Mechanically gated ion channels are hence affected, leading to a local ion fluctuation that can alter cell migration (Wei et al. 2009). Mainly, TNTs, which were described to be expressed in bovine and human mammary epithelial cells, promote cancer growth

and progression by mediating the interaction between breast cancer cells and human mesenchymal stromal cells (Pietila et al. 2013).

Despite the fact that cell junctions seem to be separate entities essential to preserve a proper tissue morphology and homeostasis, they reveal interactions overlapping localization among each other. The way different types of junctions are interlinked reveals the close interaction required for their proper formation and function. However, the proper function of cell junctions is not only dependent on successful interactions among each other, but also on their interplay and association with polarity protein that are another condition to epithelial differentiation.

2. Apico-Basal Polarity

Polarity, tight junctions and gap junction proteins act in concert whereby the same proteins that control polarity dictate the allocation of cell junction proteins. In addition, both types of proteins, apical polarity proteins and cell junction proteins, are classified as signaling hubs that regulate signal transduction pathways involved in normal and cancer cell functions (Hansen and Bissell 2000, Bazzoun, Lelievre, and Talhouk 2013). Cell junctions and polarity are major contributors to the asymmetrical aspect of cells, which is key determinant of proper tissue function. Asymmetry describes the irregular distribution of lipids, proteins and other signaling molecules across the cell (Huang and Muthuswamy 2010), in addition to the specific dispersal of organelles within a polarized cell, with differentially arranged membrane domains marking the cell's apical and basal sites. Moreover, a cell's conformation in the tissue plane is another contributor to the asymmetry (Simons and Mlodzik 2008).

The attachment of epithelial layers to the underlying basement membrane define their basal domains, while their apical poles face the luminal space. The protein and lipid differential composition of plasma membrane domains is another determinant of the apico-basolateral polarity. Specific cell markers also distinguish the basal and apical poles of a cell which reaches proper function and morphology only after successful establishment of apico-basolateral polarity (Bornens 2008). The regulation of this asymmetry and the factors underlying it are still being investigated; however, cell junctions were reported to interplay with polarity whereby AJ and TJ contribute to the formation of the apical junction complex, and also regulate events associated with the apical and basolateral domains (Tamura et al. 2008, Perez-Moreno, Jamora, and Fuchs 2003). As mentioned earlier, GJ complexes play a role in the establishment and regulation of cell polarity. Studies have shed light on the cross talk between connexins and polarity proteins; for instance, Cxs associated with ZO-1 and hence contributes to the organization of GJs {Reviewed by (Bazzoun, Lelievre, and Talhouk 2013)}. Upon Cx32 transfection into mouse hepatocytes, occludin, claudin-1 and ZO-1 protein levels are upregulated suggesting an interplay between GJs and TJs (Kojima et al. 2002). Moreover, Cx26 overexpression has been associated with elevated levels of claudin-14 in human airway epithelial cells (Morita et al. 2004). Our lab has shown for the first time that Cx43 is present in the luminal breast epithelium where it is apically localized, and that Cx43-mediated gap junction intercellular communication is vital for the establishment of apical polarity and, consequently participates in the control of the cell cycle as a mechanism to prevent cancer onset. Our lab has also revealed that the induced downregulation of Cx43 expression is associated with cell multilayering, as it is

involved in regulating the proper mitotic spindle orientation via a PI3K-dependent pathway (Submitted Bazzoun et al., 2016).

Apico-basolateral asymmetry has been thoroughly studied in the drosophila model and it was found that the assembly of core polarity proteins complexes at specific cell sites is indispensable for polarity establishment. The main complex markers of the apical epithelial cell domain include the Partitioning defective (PAR) complex that comprises PAR3, PAR6, atypical protein kinase C (aPKC) and cell division control protein 42 (CDC42), in addition to the the Crumbs (CRB) complex which constitutes the transmembrane protein CRB and its associated cytoplasmic proteins PALS1 and PALS1-associated tight junction protein (PATJ) which are together responsible of apical maintenance and regulation. On the other hand, members of the Scribble polarity complex which consists of the scribble homologue (SCRIB), discs large homologue (DLG) and lethal giant larvae homologue (LGL) are confined to the basolateral site (Ellenbroek, Iden, and Collard 2012). The establishment of apico-basal polarity in epithelial cells starts with deposition of Par3 on the cell membrane (Lemmers et al. 2004) followed by association to nectin–afadin adhesion complexes (Ooshio et al. 2007, Sakisaka et al. 2007). Afterwards, local enrichment of E-cadherin and JAMA occurs at the cell cortex indicating de novo cell–cell contacts (Adams et al. 1998, Vasioukhin et al. 2000). Consequently, Ras-related C3 botulinum toxin substrate (Rac1) is activated which inhibits RhoA subsequently leading to the formation and expansion of the cell junction complex (Nakagawa et al. 2001). Succeeding events allow for activation of cdc42 which recruits Par6 and aPKC to the membrane. aPKC in turn phosphorylates LGL and thus restricts it to the basal cell site, whereas Scribble indirectly limits apical proteins (Crumbs for instance) to the apical domain of Drosophila epithelia (Huang and

Muthuswamy 2010). Additional evidence also shows the need for E-cadherin for proper localization of the Scribble complex (Navarro et al. 2005). Our lab has previously shown that the loss of Cx43 in S1 normal human mammary epithelial cells induces scribble mis-localization and, as mentioned above, down-regulates PI3K activity and alters the downstream pathway which blocks proper mitotic spindle orientation, ultimately leading to the formation of multilayered S1 acini (Submitted Bazzoun et al., 2016).

a. Apico-Basal Polarity in Normal Development

Normal cell function and development is dependent on proper apico-basal polarity, similar to the need for proper cell junction establishment. For instance, any abnormal mislocalization or defect in polarity protein expression will lead to loss of function in epithelial cells (Naus and Laird 2010). In this scope, the effect of proper Scribble localization on cell cycle was assayed in MDCK cells where inhibition of Scribble membrane recruitment lead to cell cycle arrest at G1 phase (Nagasaka et al. 2006). Moreover, mislocalization of Scribble in Human T-Lymphotropic Virus (HTLV) resulted in a persistent pathogenesis of the virus (Okajima et al. 2008). As for neuronal cells, Scribble/β-catenin interactions were vital for the localization of synaptic vesicles in neurons (Sun et al. 2009). Polarity proteins are also implicated mediating epigenetic signatures. Interestingly, Par6-mediated MAPK pathway activation increases phosphorylation of histones H1 and H3 and consequently c-myc and c-fos over-expression {Reviewed by (Bazzoun, Lelievre, and Talhouk 2013)}.

b. Apico-Basal Polarity in Breast Cancer

Disruption and loss of epithelial cell polarity is nowadays a hallmark of breast cancer (Ellenbroek, Iden, and Collard 2012). Proper localization of polarity markers to the cell membrane was reported to partake in tumor suppressive effects mediated by cell junction protein complexes. These tumor suppressive properties include regulation of cell migration and cell cycle stages as well as control of the mammary morphogenesis (Martin-Belmonte and Perez-Moreno 2012). For instance, the loss Par3 induces H-ras dependent tumorigenesis (McCaffrey et al. 2012). Similarly, the loss of DLG1 or Scribble enables Ras-dependent invasion of mammary epithelial cells (Etienne-Manneville 2008, Dow et al. 2007). Moreover, it has been shown that DLG homologue 1 acts as a tumor suppressor through binding Cx32 and blocking G0/G1 transition (Duffy et al. 2007). Additionally, the knockdown of Scribble via RNA interference or its mislocalization in MCF10A mammary epithelial cells has led to cell polarity disruption, blocking of normal morphogenesis and promotion of transformation in 3D culture settings (Dow et al. 2007, Zhan et al. 2008) in addition to disruption of MCF10A migration due to the lack of polarization at the leading edge without affecting basal polarity (Dow et al. 2007). Our lab has also shown that Cx43 drives the establishment of apical polarity in the mammary epithelium, whereby *de novo* expression of Cx43 was accompanied with an apical localization of the protein in MCF10A acini which displayed an apical distribution of ZO-1 polarity protein (Submitted Bazzoun et al., 2016).

Interestingly, loss of apico-basal polarity is often accompanied by other oncogenic cues in cancer cases. The knockdown of hScrib in MDCK cells by silencing RNA (siRNA) enhanced cell cycle progression from G1 to S phase. Conversely, the proper localization of Scribble on the cell membrane was able to reverse this cell cycle

progression (Nagasaka et al. 2006). Moreover, Scribble mutations caused impaired cell growth and were able to synergise with Ras or erb2 overexpression which resulted in invasive neoplastic over-growth (Chatterjee et al. 2012). Scrib loss from the mammary epithelium also resulted in diminished luminal space in the formed ducts which is a typical feature of ductal carcinoma in situ (Zhan et al. 2008).

Proper tissue morphology and architecture, as well as normal mammary epithelial functions are dependent on a well-established apico-basolateral polarity. Therefore, any loss or disruption of polarity proteins would be able to prompt signs of early breast cancer stages such as inducing a multilayered phenotype, by stimulating the epithelial-to-mesenchymal transition and enabling cell migration (Chatterjee and McCaffrey 2014). However, effective cell migration has been reported to be associated with a stabilized cell adhesion and enhanced communication between cancer cells (Friedl et al. 2012, Friedl 2004). In fact, a central property of collective migration of cancer cells is coordinated polarization that is established by the rearrangement of cytoskeletal and junctional proteins at the leading edge of migrating cells to facilitate synchronized retraction at the rear end of the group (Friedl and Gilmour 2009).

D. Epithelial-to-Mesenchymal Transition

EMT refers to the transition of epithelial cells into a more invasive and motile mesenchymal-like state. It is not to be denied that EMT is a crucial hallmark of cancer, yet EMT itself has different types. It can be broadly classified into three main types; Type 1, Type 2 and Type 3 (Drasin, Robin, and Ford 2011).

EMT Type 1 is associated with developmental stages. For example, nephrogenesis illustrates the orchestration of both EMT and Mesenchymal Epithelial

Transition (MET) in order to attain proper organ formation (Micalizzi, Farabaugh, and Ford 2010). EMT Type 1 is characteristic of cell migration during embryogenesis, and is marked by the expression of genes such as SOX2, SNAI1, and SNAI2 that encode transcription factors controlling EMT (Cohen et al. 2015).

EMT Type 2 is implicated in adult tissues undergoing wound healing. For instance, keratinocyte migration necessitates partial EMT, which entails the migration of these cells in form of sheets into injured site yet while maintaining cell-cell contacts (Drasin, Robin, and Ford 2011). EMT Type 2 is also involved in tissue regeneration, and fibrosis. It is often characterized by inflammation involving the TGF-β signaling (Cohen et al. 2015).

Finally, EMT Type 3 is the oncogenic signaling mechanism. Key features of this oncogenic EMT are the down-regulation of cell-to-cell contacts and, traditionally, the replacement of E-cadherin with N-cadherin known as cadherin switch, which has been challenged by BC hallmarks including upregulation of E-cadherin. Furthermore, type 3 EMT is marked by induction of mesenchymal makers, loss of polarity proteins, and upregulation of Matrix Metallo-Proteinases (MMPs) (Scully et al. 2012) while retaining some epithelial markers.

The 3rd EMT type involves several transcription factors commonly induced by upstream oncogenic signals. They include Snail, Slug, Twist, Zinc finger E-box-binding protein 1 (Zeb1) and 2, the Smad proteins, and miRNAs among others and can either work independently from each other, or simultaneously in order to intiate EMT signaling cascades. Snail, slug, Twist and Zeb can directly repress E-cadherin transcription (Vesuna et al. 2008). EMT initiation mostly requires the activity of Snail1,

while the EMT state is maintained by Snail2, Twist and Zeb1/2 (Zheng and Kang 2014).

Several breast cancer cell lines have validated EMT signaling. For instance, Twist expression is the reason behind the spindle shape cell morphology associated with the EMT phenotype, as well as the cadherin switch from E-cadherin into N-cadherin and upregulation of Vimentin levels (Talbot, Bhattacharya, and Kuo 2012). On the other hand, the metastatic ability of MDA-MB-231 breast cells was majorly repressed by the inhibition of Twist1 signaling, which was accompanied by downregulation of N-cadherin levels (Pai et al. 2013).

Although cultured cell lines demonstrated the involvement of the EMT transcription machinery in stimulating invasive phenotype *in vitro*, *in vivo* evidence of EMT manifestation has only started to be established by the scientific and clinical communities (Bonnomet et al. 2012). This is majorly due to the fact that *in vivo* carcinomas are highly heterogeneous and EMT type 3 manifestation is incomplete, which hindered the attempts to prove it, yet evidence showing that circulating tumor cells from epithelial carcinomas display mesenchymal features is no longer debatable (Yu et al. 2013).

E. Endotoxin-Induced Inflammation and Tumor Progression Enhancement by Induced Inflammation

1. Endotoxin-Induced Inflammation

Endotoxin (ET), also referred to by its more active component lipopolysaccharide (LPS), is the main constituent of the outer wall of Gram-negative bacteria (Heine, Rietschel, and Ulmer 2001). Liberated upon the bacterial death and

breaking of its cell wall {Reviewed by (Holst et al. 1996)}, LPS is a potent immune stimulator and has intense immunostimulatory and inflammatory capacity (Kimball et al. 2003).

In mammals, ET is recognized by various cells expressing the pattern recognition receptor, TLR4, and other proteins, including LPS binding protein (LBP), cluster of differentiation 14 (CD14), and myeloid differential protein 2 (MD2) (Mani et al. 2012, Neal et al. 2006, Hornef et al. 2003).

ET is reported to simulate inflammation-like phenotype conditions in several *in vitro* and *in vivo* models. *In vitro*, ET was shown to activate primary cultures of alveolar epithelial cells (Haddad et al. 2001), umbilical vein endothelial cells (Pugin, Ulevitch, and Tobias 1993, Read et al. 1993), and bovine and rodent mammary epithelial cells (Okada et al. 1997, Boudjellab et al. 1998, Safieh-Garabedian et al. 2004), in addition to stimulating macrophages (Morris et al. 1992, Zhang et al. 1994), T cells (Mattern et al. 1994) and B cells (Weeks and Sibley 1988) from different species. *In vivo*, when introduced in the mammary gland, ET induced inflammation and mastitis in several animal models, such as sheep (Colditz 1987), goat (Dhondt, Burvenich, and Peeters 1977, Lengemann and Pitzrick 1987) and cows (Oliver and Smith 1982, Shuster, Kehrli, and Stevens 1993). It was also shown that endotoxin activated NF-kB suppressed β-casein expression and upregulated gelatinases, cytokines, NGF and nitric oxide (NO) in rodent mammary cells (Safieh-Garabedian et al. 2004, Maalouf, Talhouk, and Schanbacher 2010).

As such, endotoxin is a reliable model that has provided the basic information necessary to understand the inflammatory response in murine systems (Copeland et al.

2005) and has been exploited to induce transient systemic inflammation in healthy human subjects (Schedlowski, Engler, and Grigoleit 2014).

2. Evidence of Tumor Progression Enhancement by Induced Inflammation

Inflammation has been implicated in the initiation processes of several cancers. Thus, studying the inflammatory biomarkers as well as the multiple inflammatory cellular mediators and their downstream effects due to a chronic insult is important for understanding cancer initiation. Increased levels of DNA adduct formation, increased angiogenesis and altered anti-apoptotic signaling are believed to be the link through which inflammation facilitates cancer development (Brenner et al. 2014). Inflammation has been associated with breast cancer (Ham and Moon 2013). The inflammatory microenvironment is a major contributor to the aggressiveness and malignant progression of breast cancer through complex molecular mechanisms involving several cytokines and signaling networks. Recent studies have linked some inflammatory markers with breast cancer invasion such as MMPs. MMPs not only play an important role in the degradation of the ECM components upon cancer cell invasion and migration, but they also aid in recruiting multiple stromal cells during inflammation, including fibroblasts and leukocytes (Ham and Moon 2013).

Moreover, previous studies have described a role for ET in mediating EMT in several cancer models through the modification of NF-κB signaling pathway. In a mouse melanoma model, ET increased the expression of N-cadherin and Snail while decreasing zonula occludens-1 expression in a dose- and time-dependent manner. ET was also able to stimulate cell migration through activation of TLR4/NF-κB signal pathway. ET-induced EMT was shown to be critical for inflammation-initiated

metastasis. (Chen et al. 2012). In human breast cancer cells, other studies indicated that MDA-MB-231 and MCF-7 human cells may be induced by ET to undergo EMT, characterized by the acquisition of a mesenchymal phenotype, the appearance of vimentin, and the disappearance of E-cadherin (Huang, Chen, and Fang 2013). MDA-MB-231 and MCF-7 cells also exhibited an enhanced invasion and wound healing capabilities upon the ET induced stimulation of the TLR4 receptor, which was accompanied by an upregulation at the level of MMPs namely MMP-2 and MMP-9 in addition to vascular endothelial growth factor (VEGF), IL-6 and IL-10 production by human breast cancer cells.

CHAPTER THREE

MATERIALS AND METHODS

A. Cell Culture

1. SCp2 Culture

Low passage number (18 to 25) of SCp2 cells were used throughout. Cells were maintained in Dulbecco Modified Eagle Medium F12 (DMEM/F12) growth medium containing 1% penicillin-streptomycin (Pen-Strep), 5% Fetal Bovine Serum (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 Dulbecco's 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 (min). The cells were washed with 5 milliLiter (Simons and Mlodzik) complete media, and centrifuged at 1000x g for 10 mins, and the pellet was re-suspended in the appropriate amount of media and transferred into new culture plates for maintenance or used for other purposes.

Culture media of SCp2 cells subjected to long-term ET treatment were supplemented with either 0.1 or 0.5 µg/ml ET for one-month. ET was replenished with every change of media.

2. S1 and Cx43-KO S1 Cultures

Non-neoplastic S1 HMT-3522 human mammary epithelial cells (HMECs) (Briand, Petersen, and Van Deurs), between passages 52 and 60, were routinely maintained as a monolayer (2D culture) in chemically defined serum-free H14 medium

(Blaschke et al. 1994, Plachot and Lelievre 2004). Cell culture medium was changed every 48 hr during the culture period. Cx43-KO S1 cells were cultured in selection media consisting of serum-free H14 supplemented with with hygromycin-B (150 µg/ml; Calbiochem, San Diego, CA).

Culture media of S1 and Cx43-KO S1 cells subjected to 9-day exposure were supplemented with 10 µg/ml ET right after splitting (day zero) and ET was replenished with every change of media (every 48 hours) until media collection on day 9. However, culture media of S1 and Cx43-KO S1 cells subjected to long-term ET treatment were supplemented with 10 µg/ml ET for one-month. ET was replenished with every change of media.

For three-dimensional (3D) cell culture, S1 cells were plated on MatrigelTM (60 µl/cm2, BD Biosciences, Bedford, MA) to induce the formation of acini at a density of $5x10^4$ cells/ml in the presence of culture medium containing 5% MatrigelTM (Plachot and Lelièvre, 2004). EGF was omitted from the culture medium after day 7 to allow completion of acinar differentiation usually observed on day 8 or 9 (Plachot and Lelievre 2004, Lelièvre and Bissell 2005).

3. MDA-MB-231 and MCF-7 Cultures

Low passage number (18-30) of MDA-MB-231 (Monroe Dunaway Anderson Metastatic Breast cancer) and MCF-7 (Michigan Cancer Foundation cells number 7) were grown in humidified incubator (95% air, 5% CO2) at 37°C, in Rose Park Memorial Institute (RPMI) 1640 media (Lonza, Belgium) supplemented with 1% penicillin-streptomycin Pen-Strep as well as 10% FBS. This media was changed once every two days. When reaching 80% confluency, cells were washed with 1x PBS then

incubated with 2x trypsin at 37°C for 1 min. The cells were washed with 5 mL complete media, and centrifuged at 1000x g for 5 mins, and the pellet was re-suspended in the appropriate amount of media and transferred into new culture plates for maintenance or used for other purposes.

Culture media of MDA-MB-231 and MCF-7 cells subjected to long-term ET treatment were supplemented with 1µg/ml ET for one-month. ET was replenished with every change of media.

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

Culture media were collected from the respective cultures 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 Tris-HCL, 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 hours in substrate buffer (50 mMTris-HCL, 5 mM $CaCl_2$, 0.02%NaN_3, pH 8.0) at 37°C. The gels were stained for 2 hours at room temperature in 0.05% Comassie blue R-250 (Sigma, St. Louis,Missouri, USA), in 50% methanol and 10% acetic acid and destained in distilled deionized water for 16 hours. 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 bands were quantified using ImageJ in triplicates and data is represented as the average fold increase of MMP

band peak area (Arbitrary Basal Density) of three experiments ± standard error of the mean (SEM) (AU ± SEM).

C. Wound Healing Assay

Non-treated cells and those subjected to long-term ET treatment were plated in 6-well plates at a density of $250x10^3$ cells/ml in their respective culture media (DMEM/F12 of containing 1% Pen-Strep, 5% FBS, and 0.1% insulin for SCp2 cells and RPMI supplemented with 1% Pen-Strep and 10% FBS for MDA-MB-231 and MCF-7). Culture media of cells subjected to long-term ET treatment were also supplemented with 0.1 µg/ml ET in case of SCp2 and 1µg/ml ET for MDA-MB-231 and MCF-7 cells. After 72 hours, upon reaching full confluency, cells were washed twice with PBS 1X then supplemented with growth media containing 1% Pen-Strep as well as 1% FBS. Cells were either left as control non-treated cells or subjected to short-term ET exposure by supplementing their culture media with 0.1 µg/ml ET in case of SCp2 and 1µg/ml ET for MDA-MB-231 and MCF-7 cells. Culture media of cells subjected to long-term ET treatment were also supplemented with ET as before. A straight wound was made using a pipette tip and the same site of the wound was monitored throughout time. Pictures were taken using a fluorescent microscope and the closure of the wounded area was measured 48 hours post-wounding using ImageJ.

D. Invasion Assay

Six-well tissue-culture plates were fitted with inserts (8 µm pore size). The inserts were coated with 300µl of EHS-Growth media solution of 1:20 ratio and incubated at 37°C for 4 hours; $3x10^5$ S1 cells were seeded in the inserts. After 24 hours,

the cells were fixed using 4% paraformaldehyde in PBS 1X for 20 minutes at room temperature. The cells towards the inside of the insert were removed by using a cotton swab, and nuclei of migrated cells were counterstained with Hoechst (DAPI; 4,6-diamino-2-phenylindole) (Molecular Probes, Eugene, OR, USA) at a concentration of 0.5 µg/ml, for 10 minutes at room temperature. The insert was then cut and mounted on a microscopic slide in ProLong® Gold antifade reagent (Invitrogen Molecular Probes). The inserts were then examined by fluorescence microscopy.

E. Immunofluorescent Labeling

S1 acini from fresh 3D cultures were permeabilized with 0.5% peroxide and carbonyl-free Triton X-100 (Sigma-Aldrich) 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) prior to fixation in 4% paraformaldehyde (Sigma-Aldrich). Antibodies used are rabbit polyclonal β-catenin (1:100, Santa Cruz Biotechnology, 200 µg/ml). Donkey anti-rabbit secondary antibodies conjugated with Alexafluor 568 (red) (Invitrogen Molecular Probes, Eugene, OR) were used at the manufacturer's proposed dilution (1:2000). Nuclei were counterstained with 0.5 µg/ml Hoechst (DAPI; 4,6-diamino-2-phenylindole) (Molecular Probes, Eugene, OR, USA) and specimens were mounted in ProLong® Gold antifade reagent (Invitrogen Molecular Probes). A minimum of one hundred acini were analyzed for each immunostaining using LSCM.

- Image processing

Images of immunofluorescence labeling were recorded using laser scanning fluorescent confocal microscope LSCM (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).

F. Griess Reaction Assay of NO for NOS Activity

Non-treated MDA-MB-231 cells were plated in 6-well plates at a density of $250x10^3$cells/ml in RPMI culture media supplemented with 1% Pen-Strep and 10% FBS. After 24 hours, cells were washed twice with PBS 1X then supplemented with RPMI containing 1% Pen-Strep as well as 1% FBS. MDA-MB-231 cells were either left as control non-treated cells or subjected to short-term ET exposure by supplementing their culture media with 0.1 or 1µg/ml ET. Conditioned media were collected after 48 hours, post-treatment.

The analysis of NO was accomplished by the Griess assay that measures nitrite (the stable spontaneous oxidation product of NO) using a Griess Reagent Kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Samples were assayed in duplicate and data is represented as the average concentration of NO_2^- of three experiments ± standard error of the mean (SEM) (µg ± SEM).

G. Immunoassay of Interleukin-1β

To measure IL-1β secretion in response to ET in MDA-MB-231 cells, medium collected 48 hours post-ET treatment (see previous culture method in **F. Griess reaction assay**) were assayed by enzyme-linked immunosorbent assay (ELISA) for IL-1β (DuoSet kit; R&D Systems Inc, Minneapolis, MN) according to the manufacturer's protocol. Samples were assayed in duplicate and data is represented as the average pg IL-1β/10^6 cells of three experiments ± SEM.

H. Cell Counting Using Trypan Blue Exclusion Assay

MDA-MB-231 and MCF-7 cells were plated in 10 cm culture dishes at a density of 10^4 cells in each dish in RPMI culture media supplemented with 1% Pen-Strep and 10% FBS. Media of cells subjected to long-term ET treatment was supplemented with 1µg/ml ET. After 48 hours post-plating, the medium was replaced with RPMI containing 1% Pen-Strep as well as 1% FBS. Culture media of short and long-term ET treated cells were supplemented with 1µg/ml ET. The cells were counted from triplicates after 96 hours post plating. First, media was removed, and the cells subsequently trypsinized and collected. Cells were then diluted in Trypan Blue (1:1) ratio (V:V) and counted using a hematocytometer. Experiments were repeated at least three times.

I. Statistical Analysis

Data were presented as means ± SEM and statistical comparisons were done using Microsoft Excel. 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 $p < 0.001$.

CHAPTER FOUR

RESULTS

Non-cytotoxic endotoxin concentrations that elicit an inflammatory response without affecting cell viability, and normal cell morphology, were determined for different cell types used in these studies. As such, 0.1 µg/ml ET was used with the normal mouse mammary epithelial cells SCp2. As for the treatment of normal human mammary epithelial cells HMT3522 S1 and their tumor initiated counterpart Cx43-KO S1, a concentration of 10 µg/ml was utilized. 1 µg/ml ET was used with human breast cancer cells MCF-7 of intermediate invasiveness as well as the highly invasive human breast cancer cells MDA-MB-231.

A. ET upregulates gelatinases and enhances migration of SCp2 cells.

Bacterial ETs, lipopolysaccharides and peptidoglycans, produced during bacterial growth and lysis, are capable of activating the immune functions and inducing cytokines (El-Koraie et al. 2013), and have been used *in vitro* in various epithelial (Haddad et al. 2001, Okada et al. 1997, Boudjellab et al. 1998, Safieh-Garabedian et al. 2004) and immune cell models (Morris et al. 1992, Mattern et al. 1994, Zhang et al. 1994, Weeks and Sibley 1988), and *in vivo* (Oliver and Smith 1982, Shuster, Kehrli, and Stevens 1993) to trigger an inflammatory phenotype. Stimulating inflammation in breast cells via ET treatment constitutes a valid approach to mimic the earlier events preceding the onset of inflammatory breast cancer.

Short-term ET exposure consisted of a 48-hour treatment, referred to hereafter as short-term, after which conditioned media were collected and assayed, whereas long-term exposure consisted of a continuous ET treatment for a one-month period of time,

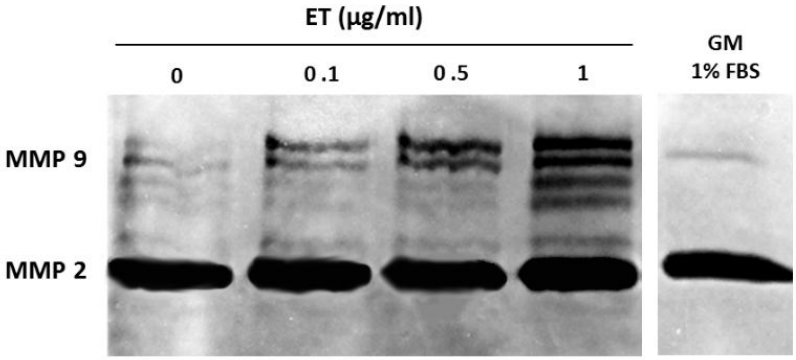
replenished with every change of media, in an attempt to mimic chronic inflammation, referred to hereafter as long-term.

To determine the effect of ET on normal mammary epithelial cells, SCp2 normal mouse mammary epithelial cells capable of differentiation upon optimal cell-cell and cell-matrix interactions, were treated with 0.1, 0.5 or 1 µg/ml ET for a short-term exposure (48 hours) after which conditioned media were assayed for MMP content.

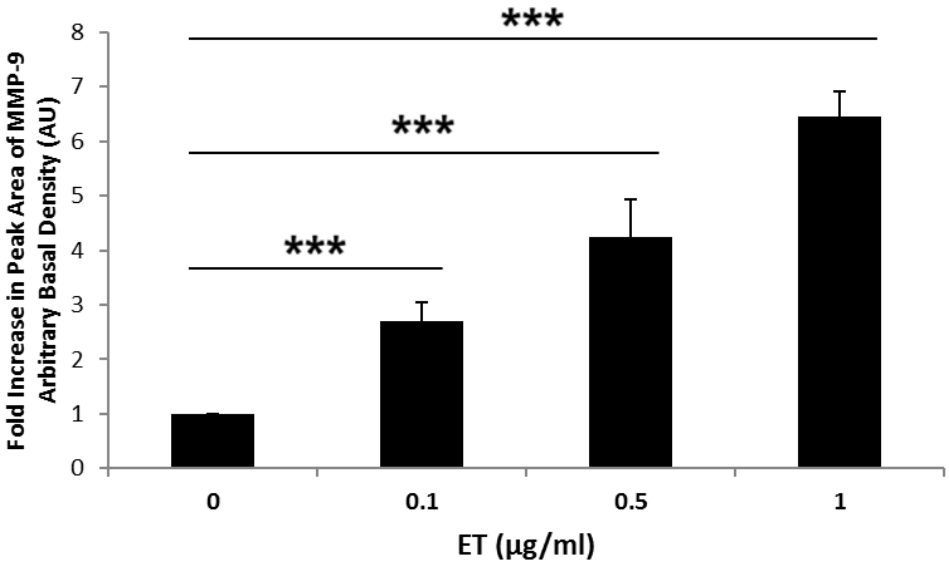
Previously, our lab has investigated the effect of 10 µg/ml ET on the normal mouse mammary cell strain CID-9 cells, which differentiate in culture and express β-casein. Results have shown that ET treatment activated NF-κB, suppressed β-casein expression and upregulated gelatinases, cytokines and nerve growth factor (NGF) (Safieh-Garabedian et al. 2004). Moreover, Maalouf et al. (2010) demonstrated that ET treatment of SCp2 cells activates NF-κB and induces IL-6 secretion and NO production (Maalouf, Talhouk, and Schanbacher 2010).

MMP-9 was upregulated when treated with increasing ET concentrations. As shown in the zymogram of Figure 1A, MMP-9 levels in conditioned media of SCp2 cells were upregulated upon treatment with 0.1, 0.5 and 1 µg/ml of ET compared to the non-treated control. Quantification of the zymography bands denotes around a two-fold increase in MMP-9 content of the conditioned media collected from SCp2 cells treated with 0.1 µg/ml of ET, a four-fold increase when treated with 0.5 µg/ml and a six-fold increase upon treatment with 1 µg/ml ET, as compared to the non-treated control cells (Figure 1B).

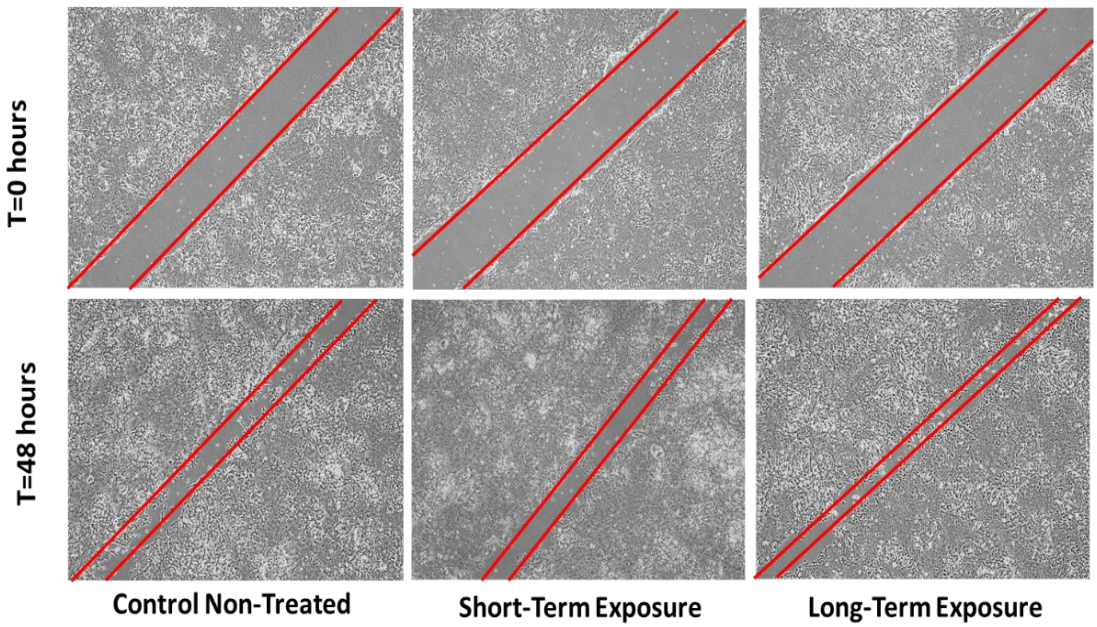
A


ET (µg/ml)
0
0.1
0.5
1
GM
1% FBS
MMP 9
MMP 2

B


***
***
***
Fold Increase in Peak Area of MMP-9
Arbitrary Basal Density (AU)
0
1
2
3
4
5
6
7
8
0
0.1
0.5
1
ET (µg/ml)

C


T=0 hours
T=48 hours
Control Non-Treated
Short-Term Exposure
Long-Term Exposure
0.1µg/ml ET

D

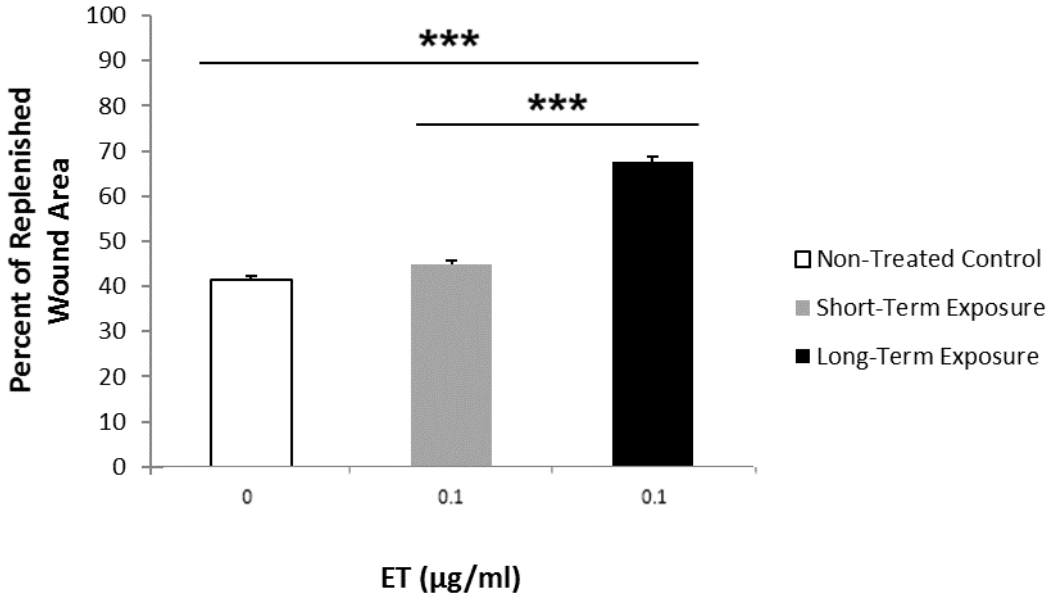


Figure 1: **Endotoxin treatment enhances matrix metalloproteinase production and cell migration of normal mouse mammary epithelial cells SCp2.** (A) Gelatin zymography of SCp2 conditioned media collected from non-treated control (0 µg/ml) and ET-treated cells (0.1, 0.5 and 1 µg/ml) 48 hours post-treatment showed an upregulation of matrix metalloproteinase MMP-9 produced by SCp2 cells. All samples were run on the same gel and under the same conditions. GM= Growth Media containing 1% FBS (B) Quantification of MMP-9 bands snown in the zymogram. Each bar represents triplicate analyses of mean ± SEM, *** $p<0.001$. (C) Wound healing assay showing enhanced replenishment of wound area with migrating SCp2 cells upon long-term exposure (one month) to 0.1 µg/ml endotoxin as opposed to the short-term exposure for 48, where the wound replenishment was not significant when compared to the non-treated control. T= 0 hours represents the wounding time when SCp2 cells reached full confluency; T= 48 hours show the same site of the wound 48 hours post-wounding. ET-treated cells showed enhanced migration. This experiment was repeated three times. Size bar = 100 µm. (D) Percent of the replenished wound area by SCp2 cells upon short and long-term exposure to 0.1µg/ml endotoxin relative to the non-treated control (0 µg/ml), 48 hours post-wounding. Cells with long-term endotoxin exposure showed enhanced replenishment of the wounded area with migrating SCp2 cells. Each bar represents triplicate analyses of mean ± SEM, *** $p<0.001$.

In order to assess the effect of long-term ET exposure on SCp2 migration compared to the non-treated control, wound healing assays were performed. Upon full confluency, a straight wound was made and the wound site was monitored throughout time. The closure of the wounded area was measured 48 hours post-wounding. Closure

of the wounded area noted in the short-term treated MDA-MB-231 cells was not significantly different when compared to the non-treated control; however, long-term exposure to 0.1 µg/ml ET, enhanced the closure of the wound compared to the non-treated control (Figure 1C) whereby 76% of the wounded area was repopulated with migrating cells, compared to only 41% in the non-treated control, 48 hours post-wounding (Figure 1D).

B. Nine-day exposure to ET upregulates gelatinases secreted by S1 and Cx43-KO S1 cells in 2D culture setting.

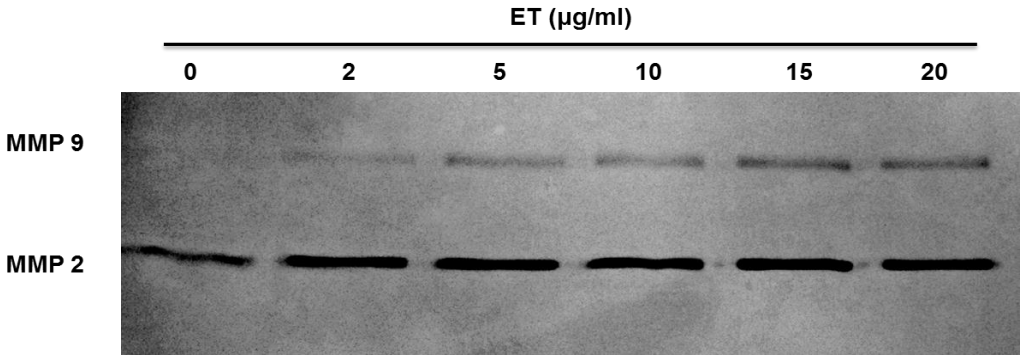
We used the non-neoplastic human mammary epithelial HMT-3522 S1 cell line (Briand, Petersen, and Van Deurs 1987), hereafter referred to as S1 cells, known to form well-differentiated polarized acini in 3D cultures, as characterized by the establishment of a basoapical polarity axis and cell cycle exit (Petersen et al. 1992, Plachot and Lelievre 2004) to examine the effect of ET on human mammary epithelial cells in 2D and 3D cultures.

Our lab has recently shown that silencing Cx43 in S1 (Cx43-KO S1) cells has a tumor promoting effect whereby it altered the cellular architecture by altering apical polarity and disrupting the gap junction complex assembly at the membrane, ultimately leading to increased proliferation and disorientation of the mitotic spindle in dividing cells (Submitted Bazzoun et al., 2016).

S1 cells in 2D cultures were treated with 2, 5, 10, 15 and 20 µg/ml ET for 9 days. Conditioned media were collected at day 9 and assayed for MMPs. MMP-9 was upregulated when treated with increasing ET concentrations. As shown in the zymogram of Figure 2A, MMP-9 levels in conditioned media of S1 cells were

upregulated upon treatment with ET compared to the non-treated control. Quantification of the zymography bands denotes a 2.3-fold increase in MMP-9 content of the conditioned media collected from S1 cells treated with 2 µg/ml of ET, a 4.5-fold increase when treated with 5 µg/ml ET, 4.9-fold increase for 10 µg/ml ET, 7.4-fold increase with 15 µg/ml ET and a 10-fold increase upon treatment with 20 µg/ml ET, as compared to the non-treated control cells (Figure 2B).

A



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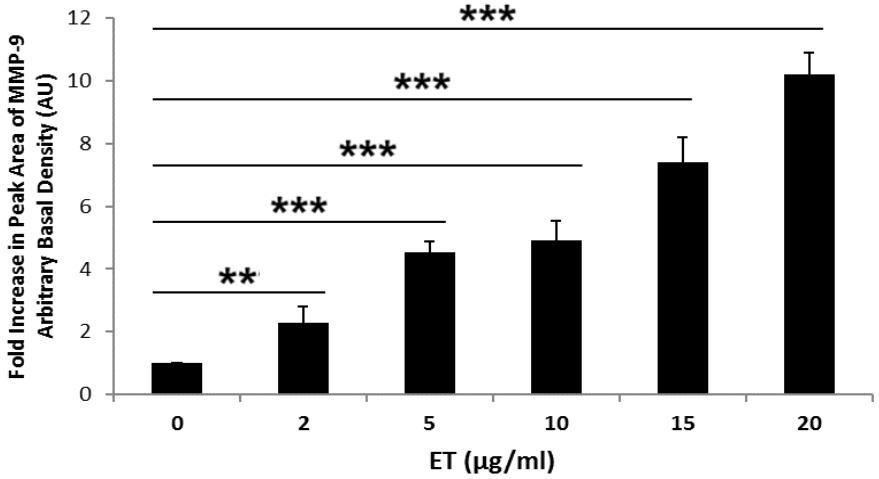


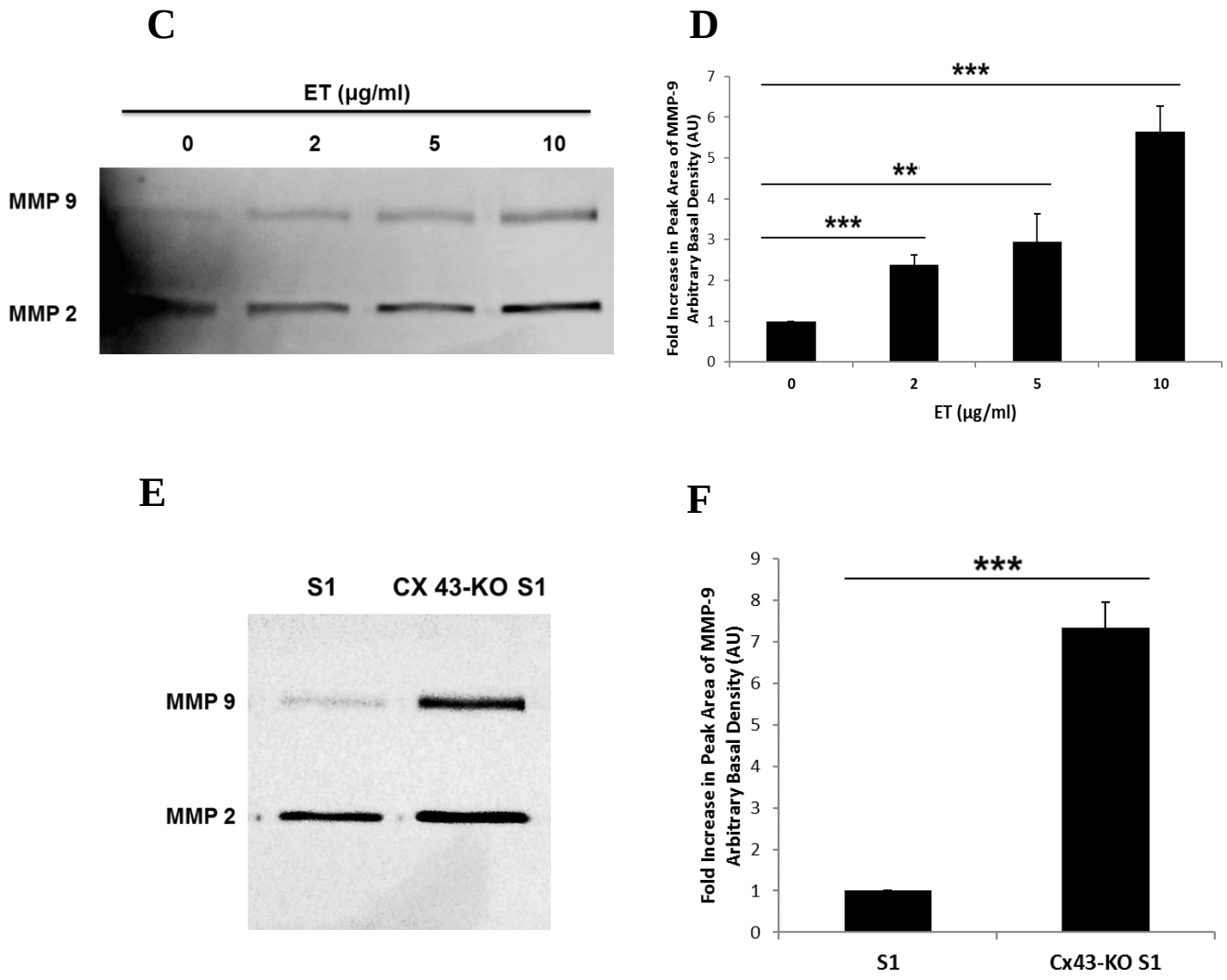


Figure 2: **Endotoxin treatment enhances matrix metalloproteinase production by S1 normal human mammary epithelial cells and the tumor-initiated Cx43-KO S1 cells.** (A) Gelatin zymography of conditioned media collected from S1 cells non-treated control (0 µg/ml), and ET-treated S1 (2, 5 and 10, 15 and 20 µg/ml) collected from 2D cultures on day 9 post-treatment showed an upregulation of matrix metalloproteinase MMP-9 with increasing endotoxin concentrations. All samples were run on the same gel and under the same conditions. (B) Quantification of fold increase in peak area of MMP-9 bands shown in the zymogram of fig. 2A. Each bar represents triplicate analyses of mean ± SEM, ** $p<0.01$; *** $p<0.001$. (C) Gelatin zymography of conditioned media collected from Cx43-KO S1 cells non-treated control (0 µg/ml), and ET-treated Cx43-KO S1 (2, 5 and 10 µg/ml) collected from 2D cultures on day 9 post-treatment showed an upregulation of matrix metalloproteinase MMP-9 with increasing endotoxin concentrations. All samples were run on the same gel and under the same conditions. (D) Quantification of fold increase in peak area of MMP-9 bands shown in the zymogram of fig. 2C. Each bar represents triplicate analyses of mean ± SEM, ** $p<0.01$; *** $p<0.001$. (E) Gelatin zymography of non-treated S1 and Cx43-KO S1 2D media collected on day 9, showed an upregulation of MMP-9 by Cx43-KO S1 compared to S1 cells. All samples were run on the same gel and under the same conditions for three replicates. (F) Quantification of fold increase

in peak area of MMP-9 bands shown in the zymogram of fig. 2E, corrected for the 30% increase in cell count observed with Cx43-KO S1 compared to S1 cells. Each bar represents triplicate analyses of mean ± SEM, *** $p<0.001$.

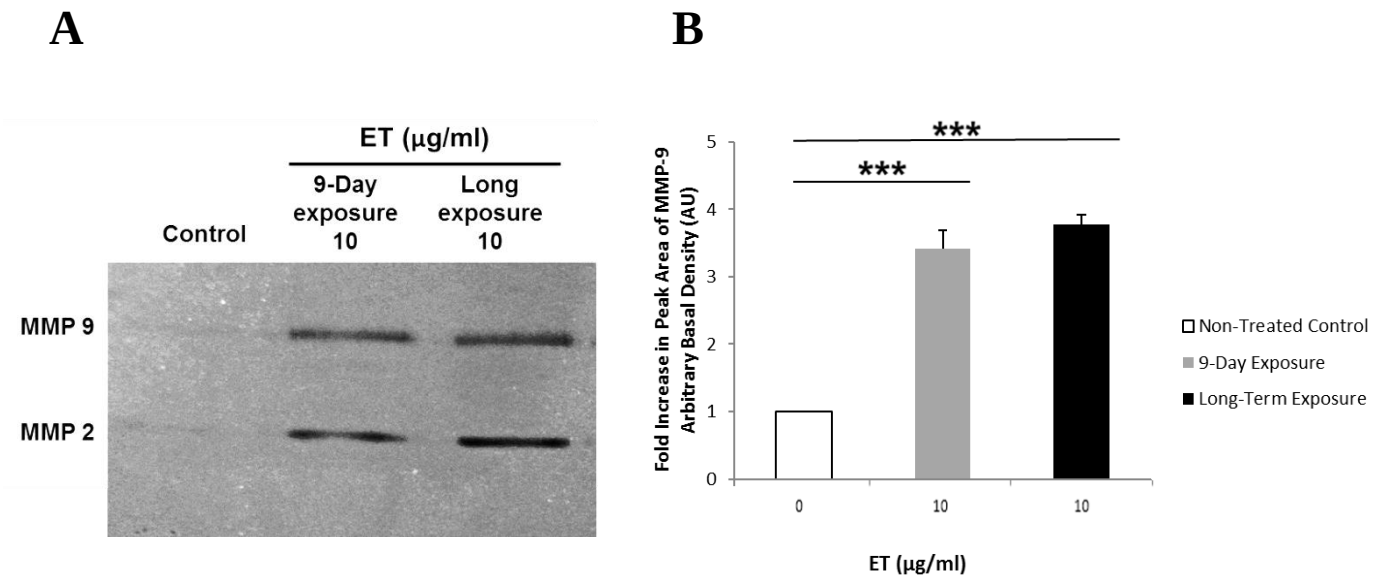
Similarly, Cx43-KO S1 cells were subjected to a 9-day treatment in 2D culture at 2, 5 and 10 µg/ml ET concentrations after which MMPs were assayed in the collected conditioned media. MMP-9 was also upregulated with increasing ET concentrations. As shown in the zymogram of Figure 2C, MMP-9 levels in conditioned media of Cx43-KO S1 cells were upregulated upon treatment with 2, 5 and 10 µg/ml of ET compared to the non-treated control. Quantification of the zymography bands denotes a 2.4-fold increase in MMP-9 content of the conditioned media collected from Cx43-KO S1 cells treated with 2 µg/ml of ET, a 3-fold increase when treated with 5 µg/ml ET, and a 5.6-fold increase upon treatment with 10 µg/ml ET, as compared to the non-treated control cells (Figure 2D).

A noteworthy observation is the higher production of MMP-9 basal levels secreted by Cx43-KO S1 as compared to those of S1 cells shown in the zymogram of Figure 2E. Since Cx43-KO S1 cells exhibit a faster rate of proliferation, we needed to correct for the difference in cell number. A 30% increase in cell growth is noted in Cx43-KO S1 cell cultures compared to S1 cells. Therefore, after normalizing to this 30% increase, we detected a 7-fold increase in MMP-9 content of Cx43-KO S1 media as compared to that of S1 cells (Figure 2F).

C. Long-term exposure to 10 µg/ml ET also upregulates the levels of MMP-9 secreted by S1 and Cx43-KO S1 cells in 2D cultures.

Chronic inflammation in S1 and Cx43-KO S1 cells was mimicked by a long-term exposure to 10 µg/ml of ET for one month. Conditioned media were collected on day 9 of culture from S1 and Cx43-KO S1 cells subjected to long-term ET treatment, and MMPs were assayed and compared to levels noted after 9-day exposure and to levels of the non-treated control.

Both of the 9-day and long-term ET exposures were capable of upregulating MMP-9 in S1 conditioned media, compared to the non-treated control (Figure 3A). Quantification of the zymography bands denotes a 3.4-fold increase in MMP-9 content of the conditioned media collected from S1 cells after 9-day ET exposure, while a 3.7-fold increase in MMP-9 was detected for cells under long-term ET exposure, as compared to non-treated control cells (Figure 3B).



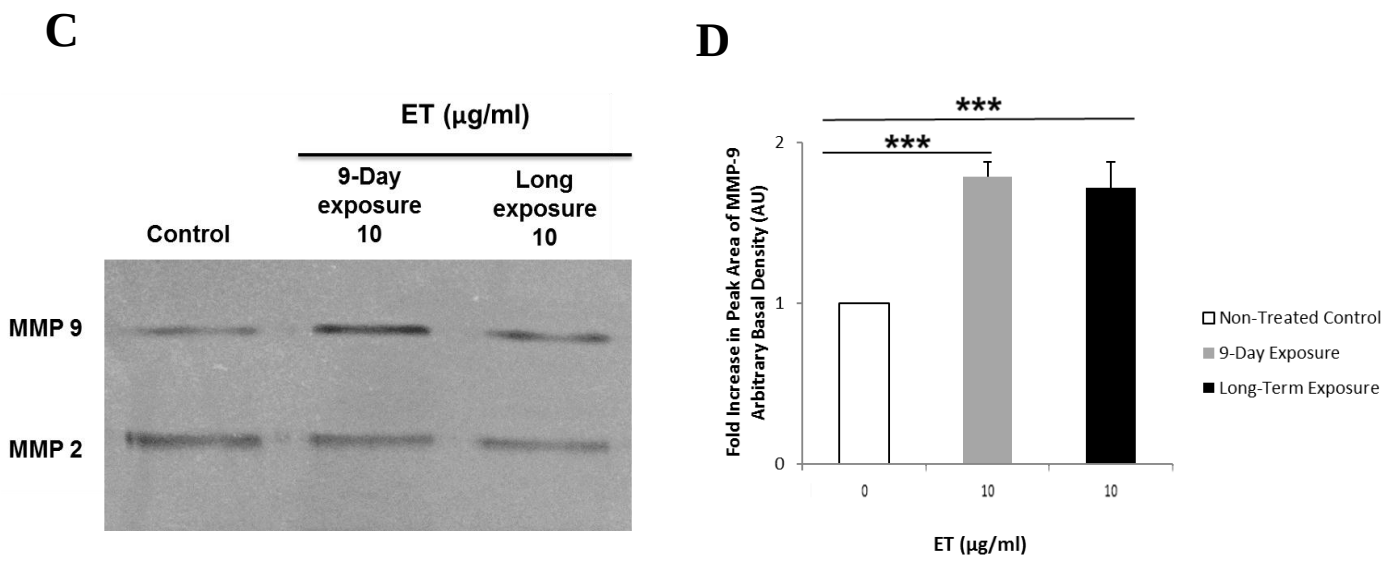


Figure 3: **Endotoxin enhances matrix metalloproteinase production by S1 normal human mammary epithelial cells and the slightly initiated Cx43-KO S1 cells upon both, 9-day and long-term exposures.** (A) Gelatin zymography of conditioned media collected from S1 cells non-treated control (0 µg/ml), and ET-treated S1 (10 µg/ml) collected from 2D cultures on day 9 post-treatment and after a long-term exposure (one month) showed an upregulation of matrix metalloproteinase MMP-9 for both exposures as compared to the non-treated control. All samples were run on the same gel and under the same conditions within fig. 3A. (B) Quantification of fold increase in peak area of MMP-9 bands shown in the zymogram fig. 3A. Each bar represents triplicate analyses of mean ± SEM, *** $p<0.001$. (C) Gelatin zymography of conditioned media collected from Cx43-KO S1 cells non-treated control (0 µg/ml), and ET-treated Cx43-KO S1 (10 µg/ml) collected from 2D cultures on day 9 post-treatment and after a long-term exposure (one month) showed an upregulation of matrix metalloproteinase MMP-9 for both exposures as compared to the non-treated control. All samples were run on the same gel and under the same conditions within fig. 3C. (D) Quantification of fold increase in peak area of MMP-9 bands shown in the zymogram fig. 3C. Each bar represents triplicate analyses of mean ± SEM, *** $p<0.001$.

Similarly, the 9-day and long-term ET exposures were both capable of upregulating MMP-9 in Cx43-KO S1 conditioned media, compared to the non-treated control (Figure 3C). Quantification of the zymography bands denotes a 1.8-fold increase in MMP-9 content of the conditioned media collected from Cx43-KO S1 cells under 9-day ET exposure, while a 1.7-fold increase in MMP-9 was detected for cells under long-term ET exposure, as compared to non-treated control cells (Figure 3D).

For S1 and Cx43-KO S1 cells, the upregulation of MMP-9 after 9-day and long-term exposures to 10 µg/ml ET was similar in both cases, and no significant difference is noted between the two types of ET exposures.

D. ET enhances S1 and Cx43-KO S1 cell invasion.

Recent studies in our lab investigating the effect of Cx43 silencing on the normal differentiated phenotype of human mammary epithelial cells showed that the loss of Cx43 was sufficient to induce cell cycle entry, promote matrigel invasion and enhance proliferation of the nontumorigenic S1 cells (Submitted Bazzoun et al., 2016). Therefore, in an effort to determine the effect of ET treatment on matrigel invasion, trans-well invasion assay was performed by seeding equal numbers of S1 and Cx43-KO S1 cells over trans-well filters (having pores of 8 µm in diameter) coated with matrigel components. Control S1 and Cx43-KO S1 cells were not treated, while ET-treated cells were either subjected to a 9-day treatment or a long-term exposure (one month) to 10 µg/ml ET.

After 24 hours, the invasion capacity of tumor initiated Cx43-KO S1 cells, where Cx43 was silenced, was first compared to the minimally invading control non-treated S1 cells. Non-treated Cx43-KO S1 cells had ~1.5-fold increase in their invasion capacity compared to the control non-treated S1 cells (Figure 4 A and B).

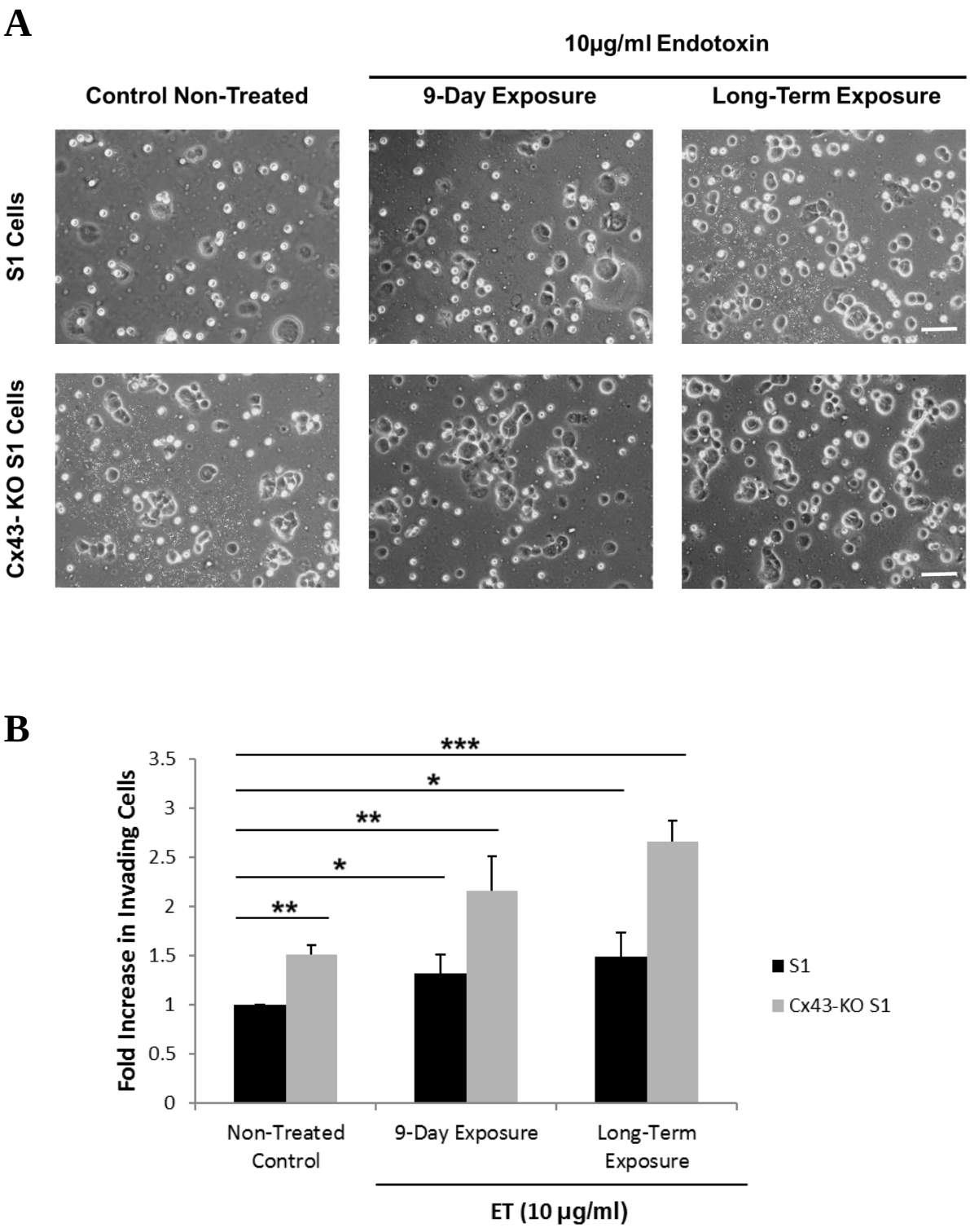


Figure 4: Endotoxin treatment enhances S1 and Cx43-KO S1 cell invasion. (A) Matrigel invasion assay showed a greater invasiveness of S1 and Cx43-KO S1 cells upon both, 9-day and long-term (one month) exposures to 10 µg/ml endotoxin. This assay was repeated 3 times. Size bar=10µm. (B) Fold increase in the number of invading ET-treated S1 and Cx43-KO S1 cells subjected to 9-day and long-term exposures to 10 µg/ml ET relative to the non-treated control (0 µg/ml). Each bar represents triplicate analyses of mean ± SEM, *p<0.05; ** p<0.01; *** p<0.001.

As for ET treatment within S1 cells, there was around 1.3-fold increase in the capacity of ET treated S1 cells to invade the matrigel, upon a 9-day exposure, whereas the long-term ET exposure resulted in ~1.5-fold increase in S1 cells invasion capacity compared to the control non-treated S1 cells (Figure 4 A and B).

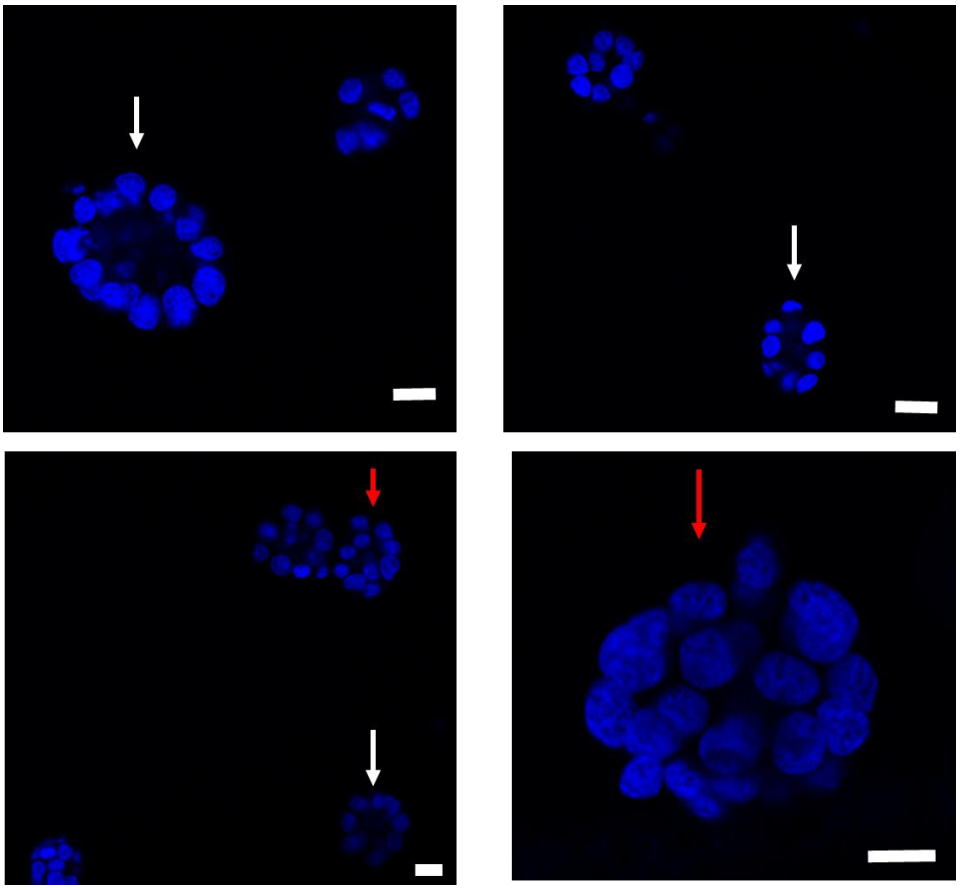
ET treated Cx43-KO S1 cells subjected to 9-day exposure showed ~2.1-fold increase in their capacity to invade matrigel, while the long-term exposure lead to ~2.6-fold increase in the invasion capacity of ET-treated Cx43-KO S1 cells compared to the control non-treated S1 cells (Figure 4 A and B).

E. ET treatment disrupts lumen formation of S1 cells.

S1 cells in 3D cultures organize as one layer surrounding a lumen, with a well-established apical polarity (Bissell et al. 2002). To determine whether ET treatment could influence this specific positioning of epithelial cells, S1 cells were subjected to a 9-day and long-term treatment at 10 µg/ml ET, in 3D culture, after which proper acinar morphogenesis and lumen formation was evaluated. Results suggested that ET treatment disrupted the acinar morphogenesis. ET treatment, whether for 9 days or long-term, altered the proper monolayer lumen of S1 cells in 3D cultures. Acinar malformation was evident from the gross morphology of acini with cells inside their lumen, in contrast to control non-treated acini that displayed typical lumen structures enclosed within a single layer of cells as depicted by the DAPI images of the control non-treated (Figure 5A) and ET-treated (Figure 5B) S1 acini. Detailed analysis showed that only ~44% of the acini in S1 cells treated for 9 days with 10 µg/ml ET had normal acinar morphology, whereas ~40% of S1 acini subjected to a long-term ET treatment

showed undisrupted lumens compared to ~64% in the control non-treated S1 cells (Figure 5C).

A



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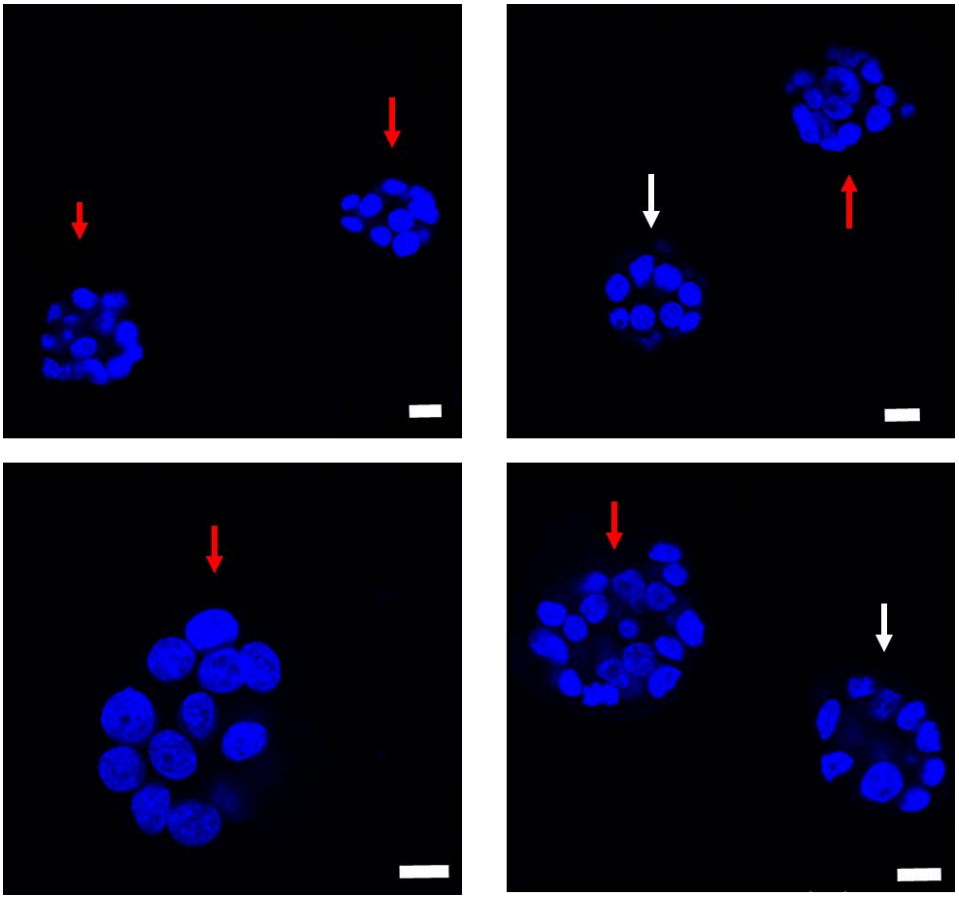

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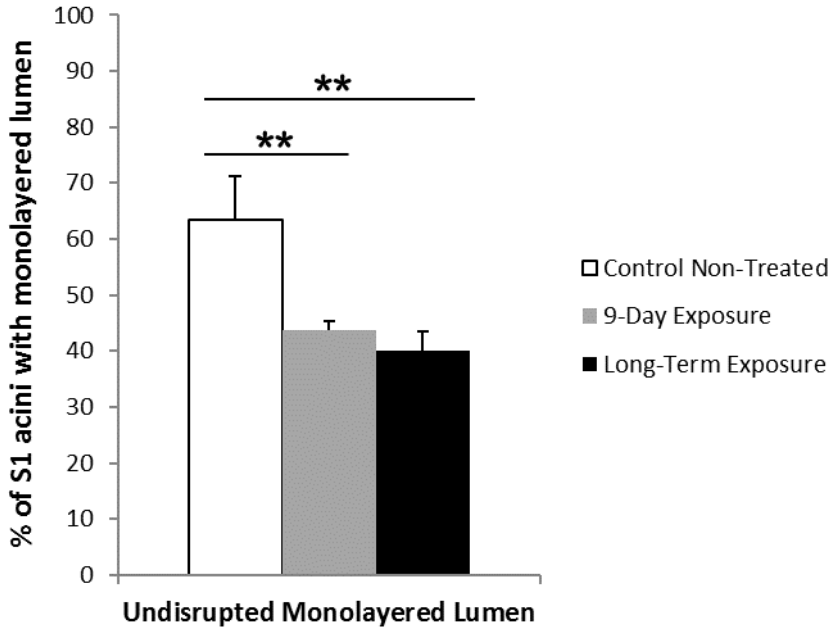


Figure 5: Endotoxin disrupts lumen formation in 3D cultures of S1 cells: Acini of (A) control non-treated S1 cells and (B) S1 cells with long-term exposure to 10 µg/ml endotoxin

stained with DAPI (blue) on day 9, and scored for lumen formation. White arrows point at acini with proper monolayered lumen while red arrows point at acini with disrupted multilayered lumen. Size bar = 10 μm. (C) The bar graph indicates the percentages of undisrupted monolayered lumen in 3D acini of control non-treated S1 cells (0 μg/ml) and S1 cells subjected to 9-day as well as long-term exposure to 10 μg/ml endotoxin. 100 acini were scored from each condition of every replicate. Each bar represents triplicate analyses of mean ± SEM, ** $p<0.01$.

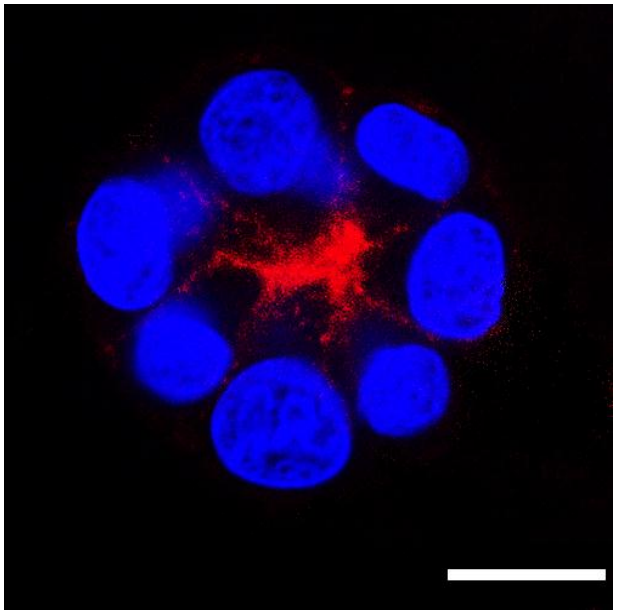
F. ET treatment disrupts β-Catenin localization in S1 acini.

Data from our lab has shown that S1 acini with a correct acinar morphology reveal an apicolateral localization of β-catenin in line with gap junction complexes. Silencing Cx43 in S1 cells induced β-catenin mislocalization, given that β-catenin associates with Cx43, acts as a signaling protein downstream of gap junctions, and its deregulation is one marker of the junctional disassembly (Talhouk 2012).

Consequently, we determined whether ET treatment would in turn induce β-catenin mislocalization. In order to check whether ET treatment triggered redistribution of β-catenin's intercellular localization, immunostaining for β-catenin was performed in control non-treated S1 acini as well as treated acini subjected to either 9-day or long-term ET treatment at 10 μg/ml. Around 60% of the control non-treated S1 acini revealed an apicolateral localization of β-catenin with a morphologically correct peripheral organization of cells around a hollow center (Figure 6A and C). The quantification of β-catenin localization pattern demonstrated no significant re-localization of β-catenin in 9-day ET treated S1 acini compared to the non-treated control, whereas it indicated a significant re-localization of β-catenin into basolateral rather than apicolateral distribution in S1 acini subjected to the long-term treatment (Figure 6B). Upon long-term ET treatment, the percentage of acini exhibiting an apicolateral expression pattern of β-catenin decreased to ~48% while the percentage of acini exhibiting a basolateral

expression pattern of β-catenin increased from ~41% in the non-treated control to ~52% in the long-term ET treated S1 acini (Figure 6C).

A



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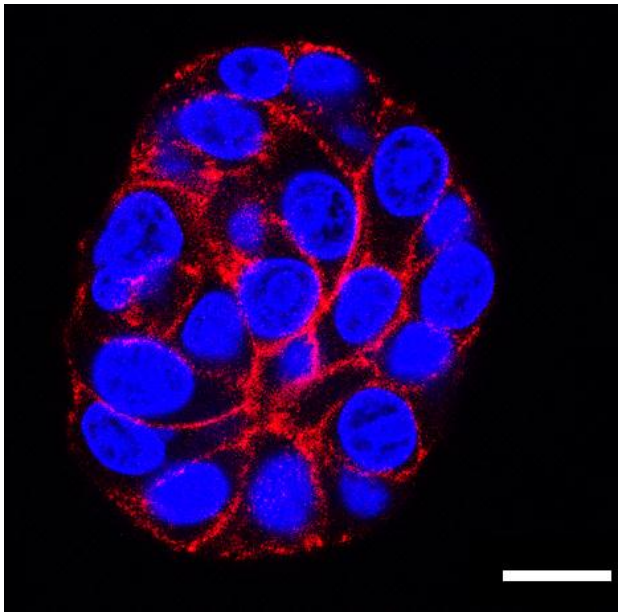

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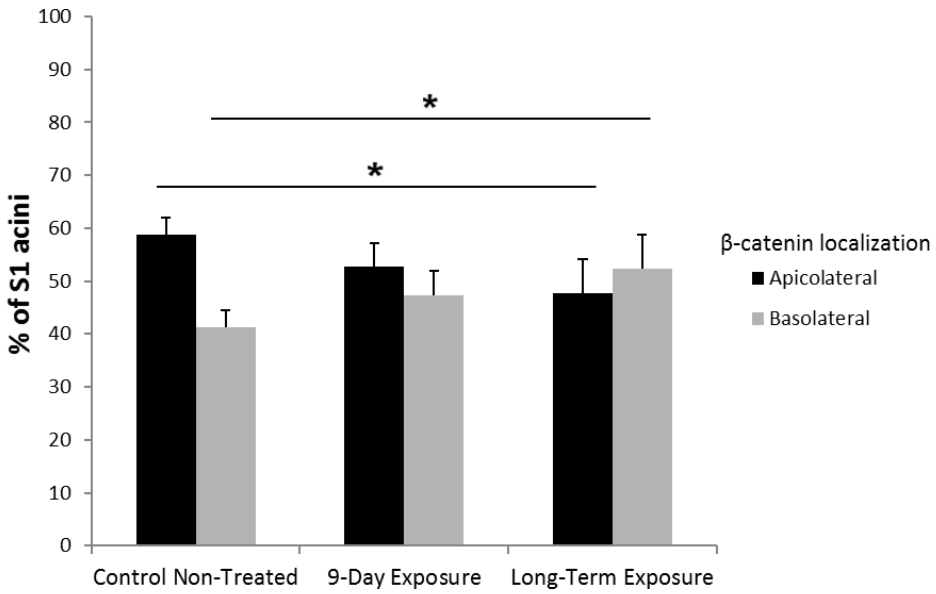


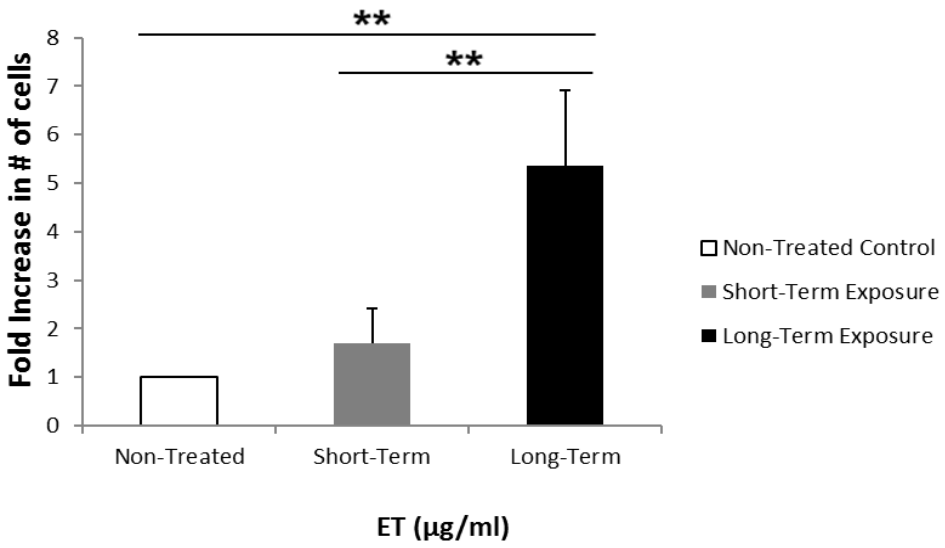
Figure 6: Endotoxin alters β-catenin localization in 3D cultures of S1 cells: Representative acinus from 3D cultures of (A) control non-treated S1 cells and (B) S1 cells subjected to long-term exposure at 10 μg/ml endotoxin stained for β-catenin (red) and counterstained with DAPI (blue) on day 9. Immunostaining revealed the mislocalization of β-catenin from the apicolateral side to basolateral side in the long-term treated S1 cells compared to the non-treated control. Size bar = 10 μm. (C) Histogram shows the quantification data of β-catenin localization. 100 acini were scored from each condition of every replicate. Each bar represents triplicate analyses of mean ± SEM, * $p<0.05$.

G. Long-term but not short-term exposure to ET enhances MCF-7 cell growth while both exposures enhance MCF-7 cell migration.

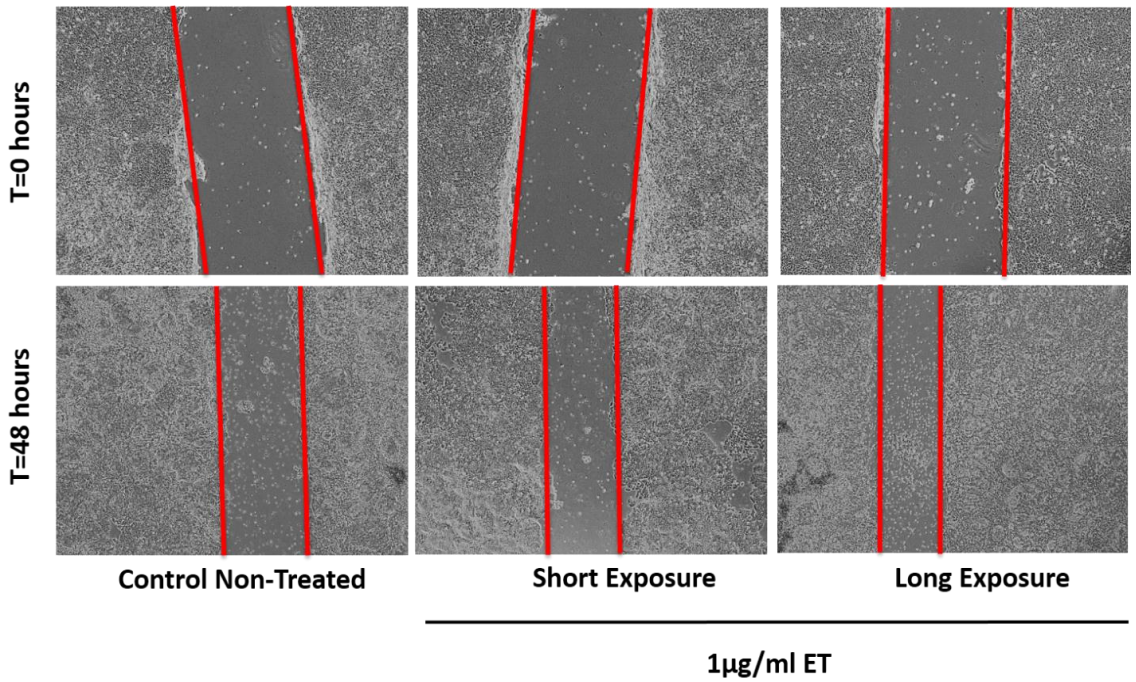
Noting the response of normal and tumor-initiated mammary cell to ET, we opted to investigate the effect of ET treatment (1 μg/ml) on human breast cancer cells MCF-7 of intermediate invasiveness. No change in the levels of inflammatory mediators namely, nitric oxide (NO), interleukin 1-β (IL-1 β), and matrix metalloproteinases (MMPs) was noted after assaying conditioned media collected from both, short (48 hours) and long-term (one month) treated MCF-7 cells.

Interestingly, long-term exposure to 1 μg/ml ET enhanced MCF-7 cell growth. Four days after plating an equal number of MCF-7 (10^4 cells/ml), short-term ET treatment did not significantly enhance the growth rate of MCF-7 cells, while the number of cells exposed to ET for a long-term was around 5 times greater than that of the non-treated control (Figure 7A).

A


**
**
Fold Increase in # of cells
8
7
6
5
4
3
2
1
0
Non-Treated
Short-Term
Long-Term
ET (µg/ml)
Non-Treated Control
Short-Term Exposure
Long-Term Exposure

B


T=0 hours
T=48 hours
Control Non-Treated
Short Exposure
Long Exposure
1µg/ml ET

C

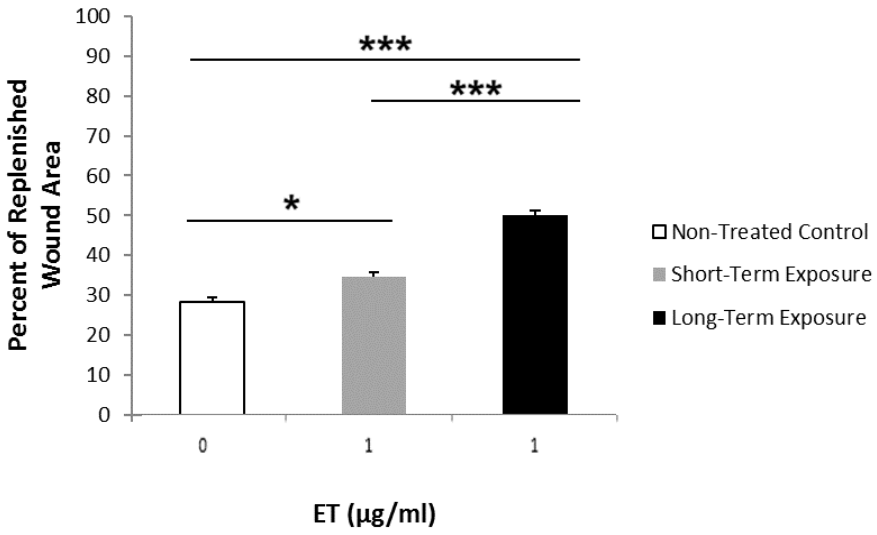

***
***
*
Percent of Replenished Wound Area
100
90
80
70
60
50
40
30
20
10
0
0
1
1
ET (µg/ml)
Non-Treated Control
Short-Term Exposure
Long-Term Exposure

Figure 7: **Endotoxin treatment increases cell count and enhances cell migration of MCF-7 breast cancer cells.** (A) Long-term exposure to 1µg/ml endotoxin (one month) increased the rate of MCF-7 cell growth, as opposed to the short-term exposure for 48 hours where the increase in cell count was not significant when compared to the non-treated control. Each bar represents triplicate analyses of mean ± SEM; ** $p<0.01$. (B) Wound healing assay showing enhanced MCF-7 cell migration upon long (one month) and short-term exposure to 1µg/ml endotoxin for 48 hours, when compared to the non-treated control (0 µg/ml). T= 0 hours represents the wounding time when MCF-7 cells reached full confluency; T= 48 hours show the same site of the wound 48 hours post-wounding. This experiment was repeated three times. Size bar = 100 µm. (C) Percent of the replenished wound area with migrating MCF-7 cells upon short and long-term exposures to 1µg/ml endotoxin relative to the non-treated control (0 µg/ml), 48 hours post-wounding. Both, the long and short-term endotoxin exposures, enhanced the rate of wound replenishment by migrating MCF-7 cells. Each bar represents triplicate analyses of mean ± SEM, * $p<0.05$; *** $p<0.001$.

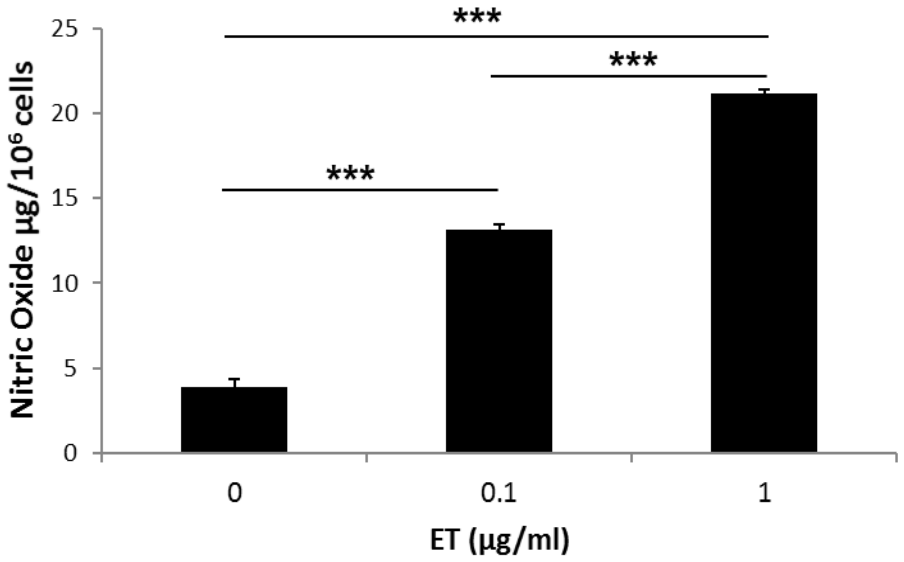
In order to assess the effect of long-term ET exposure on MCF-7 migration compared to the short-term exposure and non-treated control, wound healing assays were performed. The closure of the wounded area was measured 48 hours post-wounding as before. Both, short and long-term exposures to 1 µg/ml ET, enhanced the closure of the wound compared to the non-treated control (Figure 7B); however, upon long-term exposure, 50% of the wounded area was repopulated with migrating cells, while the short-term exposure lead to around 34% closure of the wounded area, whereas only 28% of the wounded area was closed in the non-treated control, 48 hours post-wounding (Figure 7C).

H. Short-term exposure to ET induces an inflammatory response in MDA-MB-231 cells.

After assessing the effect of Et treatment on breast cancer cells of intermediate invasiveness, the highly invasive MDA-MB-231 human breast cells were treated for a short (48 hours) or long-term duration (one month) with either 0.1 or 1 µg/ml ET. Levels of inflammatory mediators such as NO, IL1-β and MMPs, in the conditioned media were assayed after the short-term exposure.

Data showed that the level of NO detected in conditioned media from MDA-MB-231 cells subjected to short-term treatment of ET at 0.1 and 1 µg/ml, exhibited a several-fold increase compared to that detected in conditioned media of control non-treated cells. The levels of NO increased from 3.9 µg/10^6 cell in the control non-treated MDA-MB-231 cells to 13.1 µg/10^6 cell in conditioned media of cells treated with 0.1 µg/ml ET. A five-fold increase in NO level was noted in the conditioned media of MDA-MB-231 cells treated with 1 µg/ml ET, and reached 21 µg/10^6 cell (Figure 8A).

A



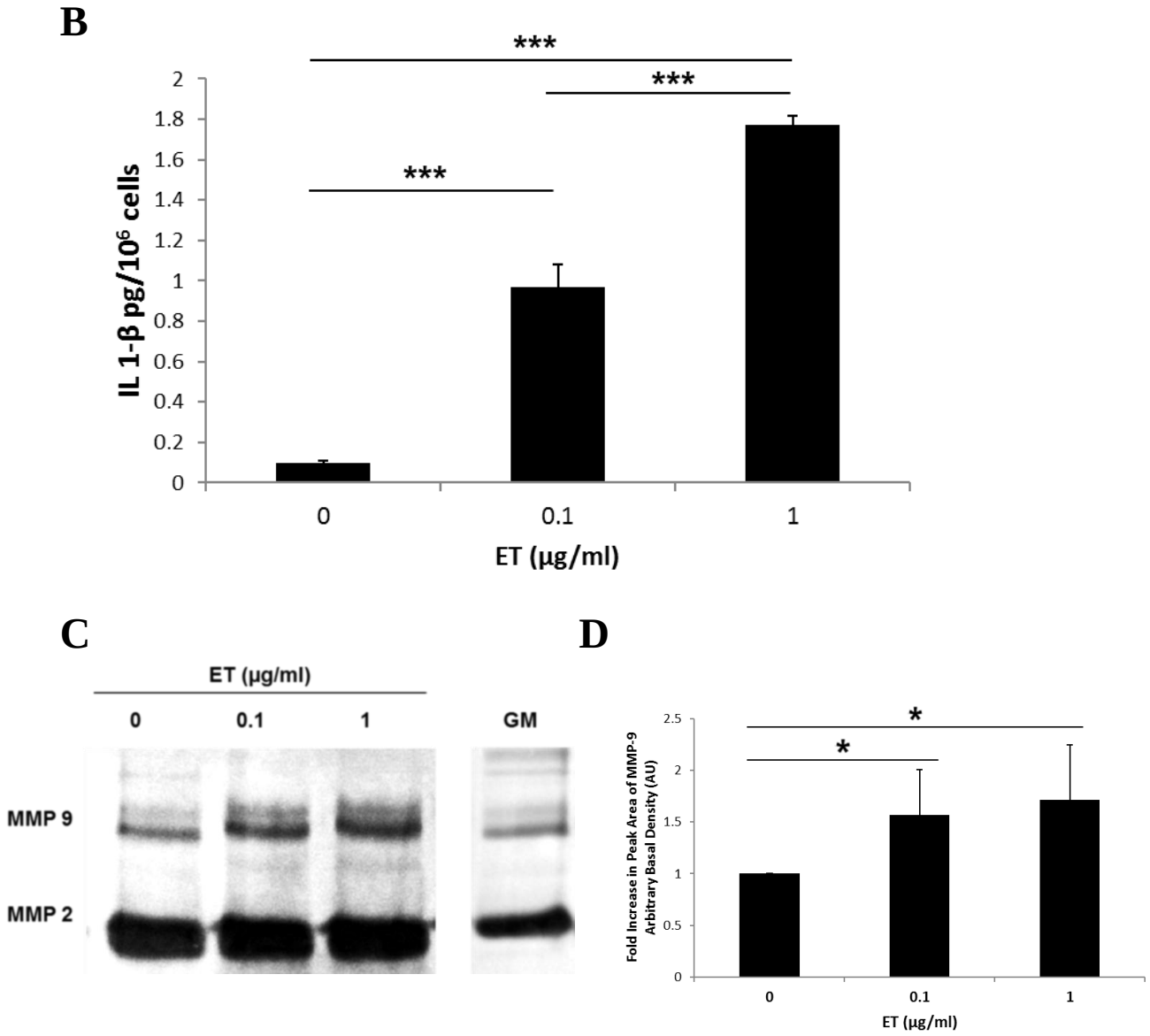


Figure 8: **Endotoxin treatment upregulates levels of inflammatory mediators produced by MDA-MB-231 breast cancer cells.** Upregulation of Nitric Oxide (A) and IL 1-β (B) levels in MDA-MB-231 conditioned media collected from ET-treated cells (0.1 and 1 µg/ml) compared to non-treated control (0 µg/ml), 48 hours post-treatment. Each bar represents triplicate analyses of mean ± SEM, *** $p<0.001$. (C) Gelatin zymography of conditioned media collected from non-treated control (0 µg/ml), and ET-treated cells (0.1 and 1 µg/ml) 48 hours post-treatment showing an upregulation of matrix metalloproteinase MMP-9 produced by MDA-MB-231. All samples were run on the same gel and under the same conditions. GM= Growth Media. (D) Quantification of fold increase in peak area of MMP-9 bands shown in the zymogram. Each bar represents triplicate analyses of mean ± SEM, * $p<0.05$.

Levels of interleukin 1-β, assayed by sandwich ELISA, indicated a six-fold increase of IL1-β in conditioned media of MDA-MB-231 cells treated with 0.1 µg/ml ET, compared to the non-treated control. Levels of IL1-β increased from 0.1 pg/10^6 cell in conditioned media of non-treated cells into 0.9 pg/10^6 cell in that of cells treated with 0.1 µg/ml ET. As for the cells treated with 1 µg/ml ET, IL1-β levels in the conditioned media was around 1.8 pg/10^6 cell, indicating an 18-fold increase compared to levels in conditioned media of non-treated control (Figure 8B).

Matrix Metalloproteinases, namely the gelatinase MMP-9 was upregulated when MDA-MB-231 cells were treated with ET. As shown in the zymogram of Figure 8C, MMP-9 levels in conditioned media of MDA-MB-231 cells were upregulated upon treatment with 0.1 and 1 µg/ml of ET compared to the non-treated control. Quantification of the zymography bands denotes around 1.5-1.7-fold increase in MMP-9 content of the conditioned media collected from MDA-MB-231 cells treated with 0.1-1 µg/ml, as compared to the non-treated control cells (Figure 8D).

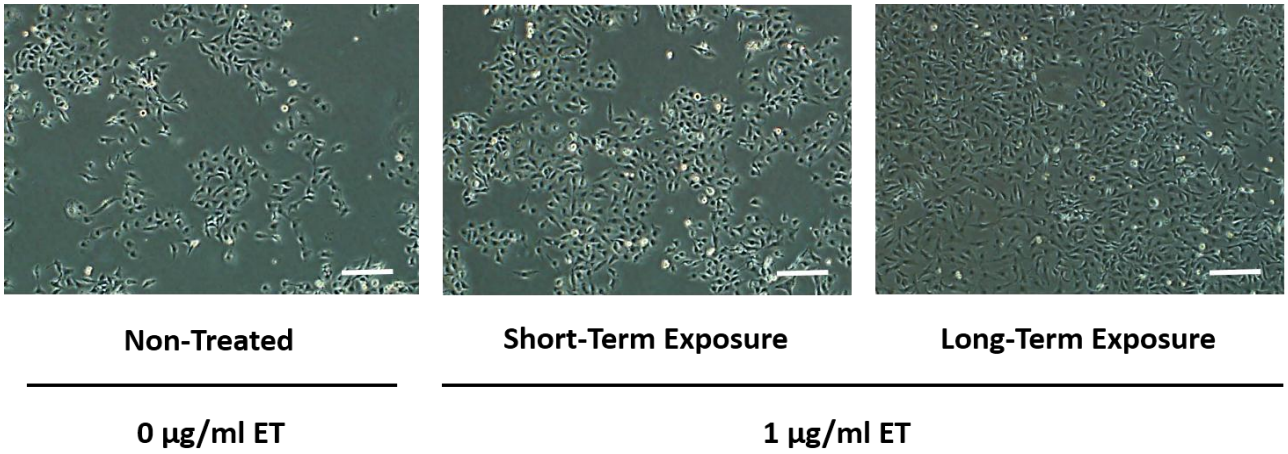
I. Long but not short-term exposure to ET enhances MDA-MB-231 cell growth and migration.

In an attempt to mimic chronic inflammation, MDA-MB-231 cells were treated for a prolonged period of time (one-month long-term exposure) with 1 µg/ml ET. ET was replenished with every media change.

Long-term ET exposure did not affect MDA-MB-231 cell morphology; however, it enhanced their growth rate and cell count (Figure 9A). Four days post-plating an equal number of MDA-MB-231 (10^4 cells/ml), the number of cells treated with ET for a short-term was not significantly different from that of the non-treated

control, while the number of cells exposed to ET for a long-term was 1.9 times greater than that of the non-treated control (Figure 9B).

A



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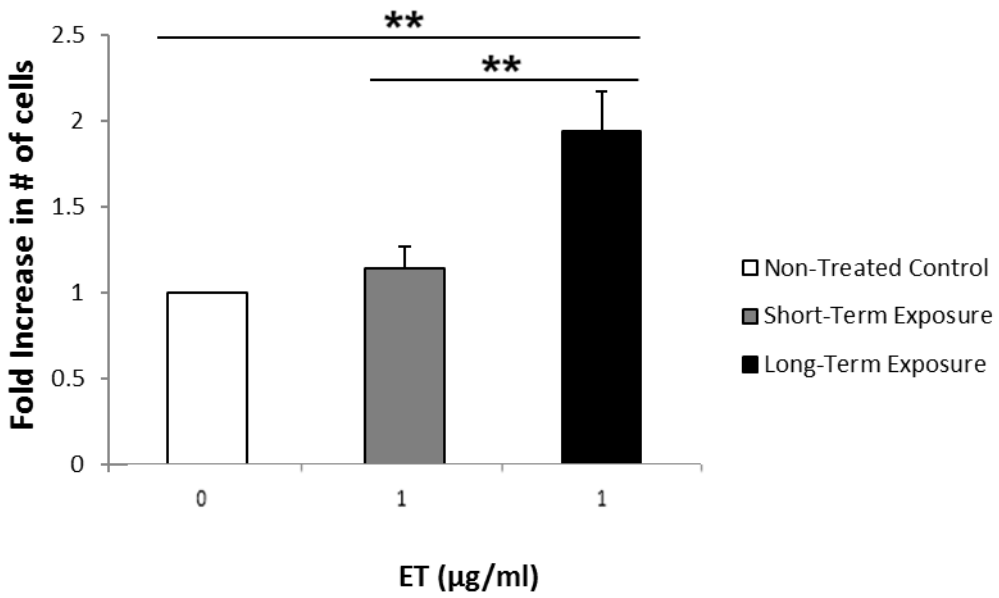

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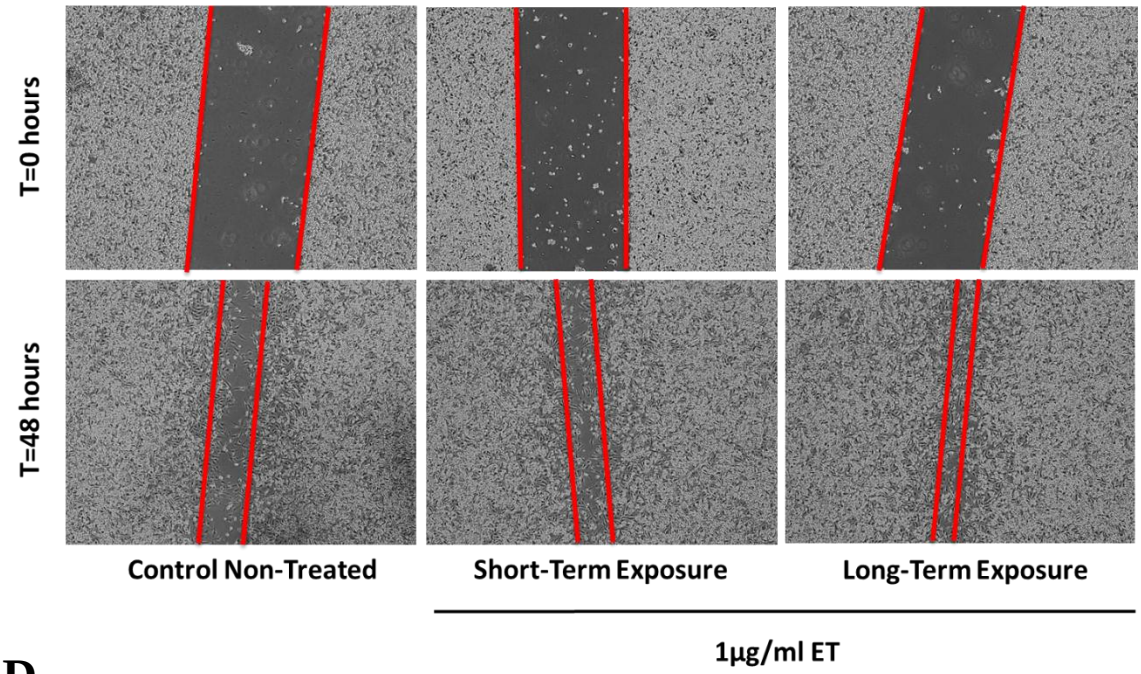

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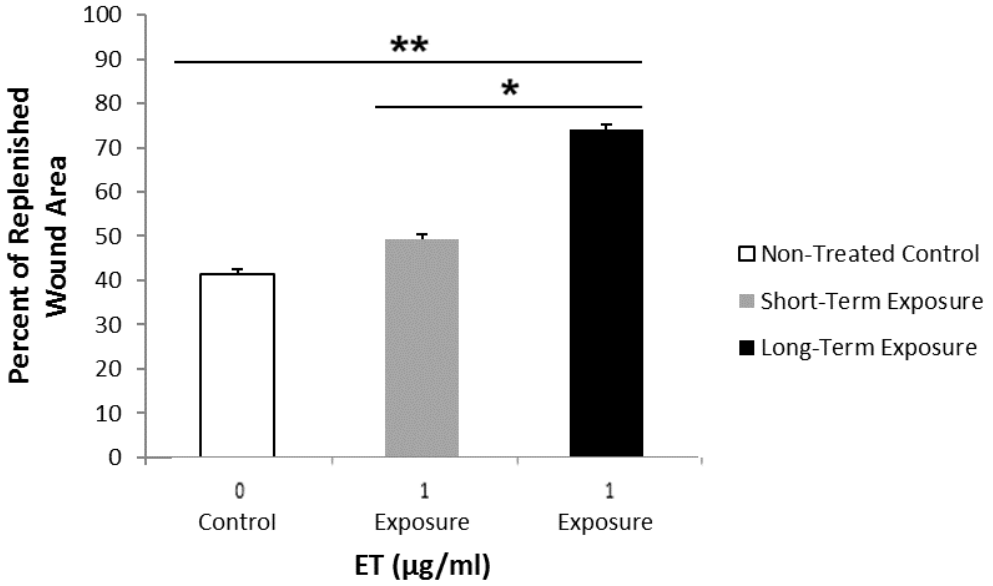


<u>Figure 9</u>: **Endotoxin treatment increases cell count and enhances cell migration of MDA-MB-231 breast cancer cells.** (A and B) Long-term exposure to 1µg/ml endotoxin (one month) increased the rate of MDA-MB-231 cell growth, as opposed to the short-term exposure for 48 hours where the increase in cell count was not significant when compared to the non-treated control. Each bar represents triplicate analyses of mean ± SEM; ** $p<0.01$. Size bar = 100 µm. (C) Wound healing assay showing enhanced MDA-MB-231 replenishment of the wounded area with migrating cells upon long-term exposure (one month) to 1µg/ml endotoxin as opposed to the short-term exposure for 48, where the wound replenishment was not significant when compared to the non-treated control. T= 0 hours represents the wounding time when MDA-MB-231 cells reached full confluency; T= 48 hours show the same site of the wound 48 hours post-

wounding. This experiment was repeated three times. Size bar = 100 µm. (D) Percent of the replenished wound area by MDA-MB-231 cells upon short and long-term exposure to 1µg/ml endotoxin relative to the non-treated control (0 µg/ml), 48 hours post-wounding. Cells with long-term endotoxin exposure showed enhanced replenishment of the wounded area with migrating cells. Each bar represents triplicate analyses of mean ± SEM, * $p<0.05$; ** $p<0.01$.

In order to assess the effect of long-term exposure of ET on MDA-MB-231 migration compared to the short-term treatment and non-treated control, wound healing assays were performed. Upon full confluency, a straight wound was made and the wound site was monitored throughout time. The closure of the wounded area was measured 48 hours post-wounding. Closure of the wounded area noted in the short-term treated MDA-MB-231 cells was not significantly different when compared to the non-treated control; however, long-term exposure to 1 µg/ml ET enhanced the closure of the wound compared to the non-treated control (Figure 9C), whereby 74% of the wounded area was repopulated with migrating cells, compared to only 42% in the non-treated control, 48 hours post-wounding (Figure 9D).

CHAPTER FIVE

DISCUSSION

Our study aims at investigating the effect of endotoxin challenge on normal, tumor initiated, and invasive human breast cells. For this reason, mammary epithelial cell cultures treated with endotoxin were used as models of inflammation. The validity of this approach stems from accumulated evidence in the literature {Reviewed by (Suffredini and Noveck 2014)}. Bacterial endotoxins, lipopolysaccharides and peptidoglycans, produced during bacterial growth and lysis, are capable of activating the immune functions and inducing cytokines (El-Koraie et al. 2013). Previously, endotoxin was reported to simulate inflammation-like phenotype conditions in several *in vitro* and *in vivo* models. *In vitro*, ET was shown to activate primary cultures of alveolar epithelial cells (Haddad et al. 2001), umbilical vein endothelial cells (Pugin, Ulevitch, and Tobias 1993), and bovine and rodent mammary epithelial cells (Okada et al. 1997, Boudjellab et al. 1998, Safieh-Garabedian et al. 2004), in addition to stimulating macrophages (Morris et al. 1992, Zhang et al. 1994), T cells (Mattern et al. 1994) and B cells (Weeks and Sibley 1988) from different species. *In vivo*, when introduced in the mammary gland, ET induced inflammation and mastitis in several animal models, such as sheep (Colditz 1987), goat (Dhondt, Burvenich, and Peeters 1977, Lengemann and Pitzrick 1987) and cows (Oliver and Smith 1982, Shuster, Kehrli, and Stevens 1993). Consequently, stimulating inflammation in breast cells via ET treatment would constitute a valid approach to mimic the earlier events preceding the onset of IBC, which was portrayed as rare, yet most lethal form of primary breast cancer targeting young women (Diessner et al. 2015). IBC diagnosis was shown to be coupled with a worse survival rate than other types of breast cancer; therefore, IBC remains a

therapeutic challenge defying the advances in treatment. Despite the data associating chronic inflammation with malignant transformation in many tissues {Reviewed by (Ham and Moon 2013)}, little is known about the effect of ET-induced inflammation on breast cancer initiation events, and whether such inflammation would trigger loss of normal morphological features of the mammary epithelium including proper lumen formation and apical polarity. Our study highlights the role of inflammatory insult on breast cancer initiation events in normal breast cells and whether such insults can "add-injury" to an already tumor-initiated or already invasive breast cells.

In this current study, our results have shown that long-term ET treatment of normal breast cells results in a possible induction of EMT phenotype. Cells from normal mouse breast epithelial model, SCp2, were able to secrete higher levels of MMPs upon short-term ET treatment. Moreover, long-term ET treatment induced faster migration of SCp2 cells. In the current study, ET concentrations were as low as 0.1 µg/ml, which is the minimal dose of ET that was capable of eliciting an inflammatory response, as indicated by the upregulation of MMP-9, without altering cell viability or normal cell morphology. The induction of EMT phenotype upon ET treatment is in line with results from previous studies conducted by our lab (Safieh-Garabedian et al. 2004) whereby the effect of endotoxin was investigated on the normal mouse mammary cell strain CID-9 cells. Such cells acquire a differentiated phenotype in response to cell–ECM, and cell–cell interaction and to lactogenic hormones (Schmidhauser et al. 1990, El-Sabban et al. 2003). SCp2 cells are subclones of CID-9: the line designated SCp2 is isolated by limited cloning from the functional, but heterogeneous CID-9 line (Desprez et al. 1993, Schmidhauser et al. 1990). SCP2 have the ability to differentiate in response to optimal cell-matrix and cell-cell interaction (El-Sabban et al. 2003). The aforementioned study

by Safieh-Garabedian et al (2004) showed that daily exposure of confluent CID-9 cells on EHS-drip to ET concentrations up to 10 µg/ml did not alter their apparent morphology nor viability. Moreover, exposure of CID-9 cells to ET induced NF-κB activation, increased gelatinase activity, inhibited β -casein expression and stimulated the production of inflammatory cytokines, such as IL-6 and TNF-α, and altered nerve growth factor production in these cells in a dose-dependent manner (Safieh-Garabedian et al. 2004). The expression of MMPs by mammary epithelial cells was previously reported to directly affect casein expression and the differentiation of the mammary gland (Talhouk, Bissell, and Werb 1992, Sympson et al. 1994). Previous studies have also shown that endotoxin treatment of SCp2 cells activates NF-κB and induces IL-6 secretion and NO production which are differentially regulated by the microenvironment of SCp2 cells in culture (Maalouf, Talhouk, and Schanbacher 2010). In light of these studies, ET insult is shown to induce an inflammatory response not only capable of upregulating the inflammatory mediators, but also of sending the normal mammary cells into a dedifferentiated state. However, inflammation in both of the aforementioned studies by Safieh-Garabedian et al. (2004) and Maalouf et al. 2010 was stimulated with 10 µg/ml ET whereas ET concentrations in our current study were as low as 0.1 µg/ml. when determining the non-cytotoxic ET concentrations, concentrations between 1 and 10 µg/ml were able to elicit an inflammatory response without altering the apparent growth and morphology of SCp2 cells; however, ET concentrations in this range slowed down the migration rate of ET treated SCp2 cells as compared to the non-treated control in wound-healing assays (data not shown). Non-treated cells were faster than those treated with ET concentrations greater than 1 µg/ml to repopulate the wounded area, while SCp2 cells treated with 0.1 µg/ml for a long term

showed an enhanced wound closure. A possible factor behind this discrepancy would be the long-term ET treatment to which SCp2 cells under study were exposed. Since short-term ET exposure did not significantly alter the migration rate of SCp2, prolonged exposure to low ET concentrations would possibly be the reason behind altering SCp2 migration rate, without affecting their apparent morphology. In this regards, studies by Yang et al. (2013) on human skin fibroblasts showed that ET might convert normal skin fibroblasts to hypertrophic scar tissue fibroblasts that participate in the formation of hypertrophic scar; hence, appropriate ET concentration may have no effect or be beneficial to skin wound healing, whereas excessive ET concentrations may delay the time of wound healing (Yang et al. 2013). Three different possibilities may explain the difference in SCp2 migration rate between short-term versus long-term treatment: selection for fibroblasts, epithelial-to-mesenchymal transition, or enhancement of cancer stem sell populations. First, prolonged exposures to ET could be selecting for the fibroblasts or specific SCp2 cell subpopulations. Vimentin expression levels in the cultured cells may be of a help in order to determine whether the cultures were enriched with fibroblasts. The other hypothesis is that long-term ET treatment can induce a mesenchymal phenotype in SCp2 cells. Recent studies showed that EMT induced by inflammatory stimuli confers to cancer cells some mesenchymal stromal cell-like immune-modulatory properties, which could be a cue to decipher cancer progression and metastatic dissemination by favoring immune escape (Ricciardi et al. 2015). And last, long-term ET treatment might enrich SCp2 cultures with stem cells, resulting in faster wound healing. This could be tested either by sphere formation assays, or through assessing the expression levels of stem cell markers mainly a low ratio of $CD44^{+}CD24^{-}$ and high levels of ALDH1 (Currie et al. 2013). Recently, pathways involved in

inflammation and wound healing have been reported to enhance cancer stem cell (CSC) populations (Arnold et al. 2015). These possibilities require further testing in order to draw a clear explanation of the enhanced migration rate seen with cells subjected to long-term but not short-term ET treatment.

The effect of ET treatment was also assessed on S1 cells, human mammary epithelial cells known to form well-differentiated alveolar-like structures known as acini in 3D cultures, and characterized by the establishment of a basoapical polarity axis and cell cycle exit (Petersen et al. 1992, Plachot and Lelievre 2004). These cells have been previously used by Plachot et al. (2009) to study mammary gland function and polarity. The use of S1 cells as a model system to study the differentiation of the mammary epithelium, is supported with further studies that showed how it recapitulates the *in vivo* characteristics. S1 cells in 3D cultures recapitulate the formation of hemidesmosomes with α-6 integrins that are observed *in vivo* and central for the establishment of basal polarity (Koukoulis et al. 1991, Weaver et al. 1997). Our lab has re-confirmed the localization of basal and apical polarity markers, and it was found that immunostaining of α-6 integrin revealed a basal localization in S1 acini whereas the core tight junction protein, ZO-1 was compartmentalized to the apical side of S1 cells, against the lumen (Submitted Bazzoun et al. 2016). This work was in line with studies conducted by Plachot et al. (2009). Interestingly, previous studies in our lab showed that Cx43 in S1 acini regulates apical polarity which is a major barrier against tumor initiation, and therefore, its loss results in the disruption of cell architecture rendering the mammary epithelium prone to tumorigenic phenotypic changes. Cx43-KO S1 acini where Cx43 is silenced by shRNA, displayed a disrupted apical polarity (Submitted Bazzoun et al. 2016), enhanced proliferation reported by Bazzoun et al. (submitted, 2016), in addition

to an enhanced potential to invade matrigel (Submitted Bazzoun et al. 2016). Therefore, Cx43-KO S1 cells recapitulate the tumor-initiated state of mammary epithelial cells.

The current study showed that short and long-term ET treated 2D cultures of S1 cells exhibit upregulated levels of MMPs. Long-term ET treatment of S1 acini not only altered the localization of β-catenin, but also disrupted the formation of a proper monolayer lumen in 3D culture and enhanced the invasion rate of S1 cells through matrigel. On the other hand, ET treatment upregulated MMPs in Cx43-KO S1 cells, in addition to enhancing their invasion rate, thereby adding-injury to the tumor-initiated Cx43-KO S1 cells.

Thinking through the proper lumen formation process, the mechanism of mitotic spindle orientation in the mammary gland has not been well characterized, yet it is demonstrated that luminal cells divide within the plane of the epithelium (Villegas et al. 2014). In order to explain the lumen disruption events, two scenarios are possible. Starting with the first possibility, several studies showed that cell-ECM interactions and in particular, those mediated by β1-integrins, play an important role in the organization and orientation of the mitotic spindle (Fernandez-Minan, Martin-Bermudo, and Gonzalez-Reyes 2007). In this light, lumen disruption of the normal S1 human breast cells could be linked to ECM alteration by the induced production of MMP-9. Normally, ECM reshaping is important for the development of normal mammary gland which undergoes various changes, including ductal development, lactation and involution (Gjorevski and Nelson 2011). This process requires the disruption and subsequent re-synthesis of ECM components through secretion of MMPs (Werb et al. 1996). MMPs exhibit a localized and tightly regulated enzymatic remodeling of stromal tissue in normal breast cells (Geho et al. 2005); however, increased levels of MMPs

have been reported in breast tumor cells, as well as in the surrounding non-cancerous breast tissue (Lebeau et al. 2004). MMPs degrade ECM proteins to allow ductal progression through the basement membrane {Reviewed by (Davies 2014)}. The subsequent loss of proper cell-ECM cues results in lumen disruption (Itoh et al. 2007, Blatchford et al. 1999). Importantly as well, Beliveau et al. (2010) demonstrated that induction of MMP9 in human nonmalignant breast cells led to loss of tissue polarity, and reinitiated proliferation. Conversely, inhibition of MMP9 with small molecule inhibitors or shRNAs restored the ability of cancer cells to form polarized quiescent structures (Beliveau et al. 2010).

Alternatively, the second scenario leading to lumen disruption is gap junction-dependent and involves a PI3K pathway. Cx43 interacts with β-catenin, a versatile protein with adhesive and transcriptional functions, highly involved in regulation of cell growth and proliferation. In addition, binding of E- or N-cadherins (E- or N-) to β-catenin is crucial for the inhibition of cell proliferation; thus, Cx43 and cadherins seem to regulate β-catenin signaling by sequestering it at the membrane (Gottardi, Wong, and Gumbiner 2001, Kamei, Toyofuku, and Hori 2003). Therefore, the mislocalization of β-catenin is considered as one of the markers of the junctional disassembly. On the other hand, cell junctions play a role in spindle orientation during early epithelial development, and their disruption could affect the orientation of the mitotic spindle {Reveiwed by (Ahringer 2003)}. The orientation of the mitotic spindle prior to cell division is essential for the maintenance of monolayered acini: this is accomplished through Par3/Cdc42-GTP complex which activates aPKC at the apical surface, which in turn blocks the NuMA/LGN complex (LGN is adaptor protein Leu-Gly-Asn repeat-enriched that partners with Nuclear organizing Mitotic Apparatus protein (NuMA))

from localizing at the apical surface, thereby preventing vertical alignment of the spindle (Hao et al. 2010). In fact, GJ-compromised and Cx43-lacking acinar structures show more random orientation of the mitotic spindle axis. Cx43-mediated GJ affect the mitotic spindle orientation through a PI3K-dependent pathway, suggested by our lab (Submitted Bazzoun et al., 2016), where Cx43 regulates the enzymatic activity of PI3K that phosphorylates Cdc42. In turn Cdc42 activates aPKCζ to exclude the NuMA complex away from the apical sides of the membrane towards the basolateral domains. Consequently, junctional disassembly marked by β -catenin re-localization, is a direct player in the process of lumen disruption.

The relationship between inflammation and Cx43 expression is very paradoxical, and their interaction in normal mammary epithelial cells is not yet resolved. Several studies have investigated the effects of endotoxin-induced inflammation on Cx43 expression in different tissues. Whereas endotoxin treatment downregulates the expression level of Cx43 in nasal epithelial cells (Yeh et al. 2005), rat neonatal astrocytes (Liao et al. 2010), and rat heart (Fernandez-Cobo et al. 1999), Cx43 expression increases after induction of inflammation in the lung (Kandasamy et al. 2015), endothelial cells of pulmonary arteries or veins (O'Donnell et al. 2014, Zhang et al. 2015), liver (Balasubramaniyan et al. 2013), and kidney cells of humans and rodents (Hillis et al. 1997, Yaoita et al. 2002). Hence, it is not clear whether ET treatment affects S1 cells normal morphology and development through altering the levels of Cx43 or through other Cx43 independent pathways.

In order to determine whether ET treatment could further initiate and EMT phenotype in S1 and Cx43-KO S1 cells, EMT markers and regulators were assayed by real-time PCR of cDNA obtained from 2D and 3D cultures of long-term treated S1 and

Cx43-KO S1 cells (see Appendix). Preliminary results indicate that long-term ET treatment upregulates the RNA levels of Snail1, Zeb1, Twist1, E-cadherin and Vimentin in 2D cultures of S1 cells, and 3D cultures of Cx43-KO S1 cells, as compared to the non-treated controls (Appendix Figure 1). Therefore, ET-induced inflammation drives S1 and Cx43-KO S1 cells into a less differentiated state through epithelial-to-mesenchymal transition.

Last, we investigated the effect of ET on moderately and highly invasive human breast cancer cells. Our results showed that long-term ET treatment enhances the cell growth and migration of MDA-MB-231 and MCF-7 cells. Our findings are in line with previous study by Trivanović et al. (2016) who demonstrated that indirect co-culture of MCF-7 and cytokines-primed mesenchymal stem cells from human adipose tissue (hASCs) stimulated EMT in MCF-7 cells. Conditioned media from inflammatory cytokines-primed hASCs were capable of enhancing invasion and migration events in MCF-7 Cells via the transforming growth factor-β1 (TGF-β1) (Trivanovic et al. 2016). Our results go hand-in-hand with a study by Hong et al. (2015) where they demonstrated that ET treatment induces EMT-like phenotypic changes, including upregulation of N-cadherin expression in MDA-MB‑231 cells, leading to enhanced cell migration and invasion. Supportive data were also found in a study by Yang et al. (2014) who demonstrated that toll-like receptor 4 (TLR4) prompts human breast cancer cells invasiveness, namely MCF-7 and MDA-MB-231 cells, via ET stimulation and is overexpressed in patients with lymph node metastasis (Yang et al. 2014). Inflammation also accelerated breast cancer progression in mouse models of breast cancer (Laoui et al. 2011). In parallel to these results, a recent study revealed that chemotherapy-induced inflammation is one of the main contributors to chemo-resistance and metastasis, using

both syngeneic and xenograft breast cancer preclinical models (Acharyya et al. 2012). Moreover, ET-activated stromal fibroblasts isolated from colon cancer induced tumor angiogenesis (Nagasaki et al. 2014). Chronic inflammation has been also associated with the development of various cancers in digestive organs {Reviewed by (Chiba, Marusawa, and Ushijima 2012)}. In xenograft lung metastasis model, ET administration enhanced lung metastasis in mice (Li et al. 2012).

In conclusion, we propose that ET-induced inflammation drives mammary epithelial cells to a less differentiated state. Our study is unique in testing the effect of long-term ET treatment on 3D cultures of normal human breast cells and our findings highlight the role of inflammatory insult on inducing breast cancer initiation events in normal breast cells. We also suggest that such insults can "add-injury" to an already tumor-initiated or already invasive breast cells. Our long-term aim is to identify inflammatory signals driving early cancer development which can be used as targets for cancer immune prevention as well as emerging biomarkers for cancer screening and early cancer detection.

APPENDIX

CHAPTER A-I

MATERIALS AND METHODS

A. Cell Culture

1. S1 and Cx43-KO S1 3D culture systems

For three-dimensional cultures, we used the Growth Factor Reduced Matrigel obtained from BD Biosciences (BD No. 354230). Matrigel (BD Biosciences, Discovery Labwork, Two Oak Park, Bedford, MA) was thawed on ice overnight at 4°C and stored in 1mL aliquots in -80°C. 35 millimeter (mm) culture dishes were placed on ice block in the biosafety cabinet, coated with 500 µl of growth factor-reduced Matrigel, then incubated at 37°C for 30 min to solidify measuring approximately 1-2 mm in thickness. While the Matrigel is solidifying, cells were counted and diluted in complete media with 2% Matrigel, to achieve a final concentration of 25,000 cells/mL. Cells were supplemented with fresh complete media with 2% Matrigel every two days. Clusters start to form by day 3, and cells were kept in culture for 8 consecutive days.

B. RNA Extraction and qPCR

1. RNA Extraction

Total RNA was extracted from cells using TRI reagent (Sigma-Aldrich, T9424) according to the manufacturer's instructions.

2. Quantitative real-time PCR (qRT-PCR)

1 µg of the total RNA extracted from cells was reverse-transcribed to cDNA using Revertaid 1st strand cDNA synthesis kit (Fermentas, Grand Island, NY). qRT-PCR was performed using iQSYBR Green Supermix in a CFX96system (Bio-Rad Laboratories, Hercules, CA). Products were amplified using primers for Snail, Zeb-1, Twist-1, E-cadherin and Vimentin as shown in Appendix-Table 1 below. To quantify changes in gene expression, the ΔCt method was used to calculate the relative-fold changes normalized to GAPDH.

Target gene	Primer	Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)
GAPDH	Forward Reverse	AAGGTGAAGGTCGGAGTCAAC GGGGTCATTGATGGCAACAATA	58	102
Snail	Forward Reverse	TCGGAAGCCTAACTACAGCGA AGATGAGCATTGGCAGCGAG	63	140
Zeb-1	Forward Reverse	TTACACCTTTGCATACAGAACCC TTTACGATTACACCCAGACTGC	58	100
Twist-1	Forward Reverse	GGGCGTGGGGCGCACTTTTA CGCTGCCCGTCTGGGAATCA	66	70
E-cadherin	Forward Reverse	CGAGAGCTACACGTTCACGG GGGTGTCGAGGGAAAAATAGG	58	119
Vimentin	Forward Reverse	AGTCCACTGAGTACCGGAGAC GGTTCCTTTAAGGGCATCCAC	58	65

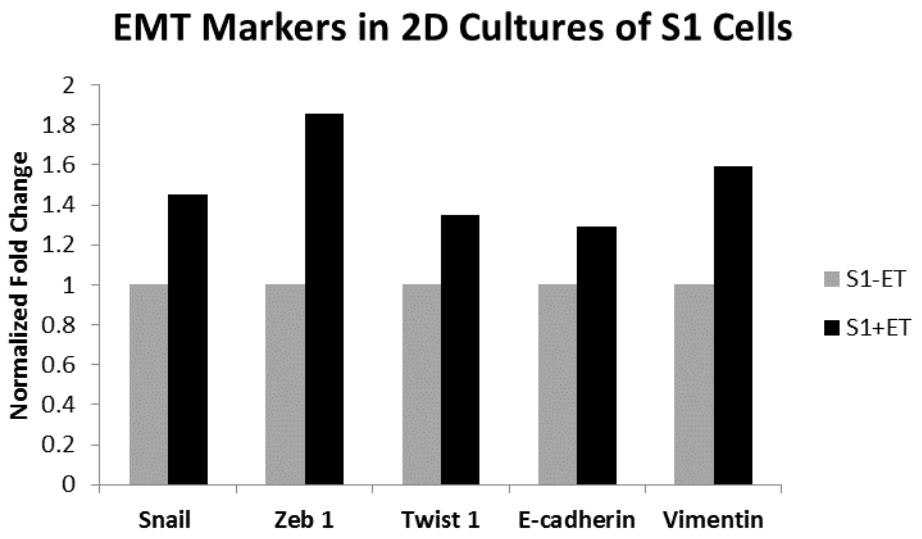
Appendix-Table1: Table showing different target genes with respective forward and reverse sequence, annealing temperature and amplicon size

APPENDIX

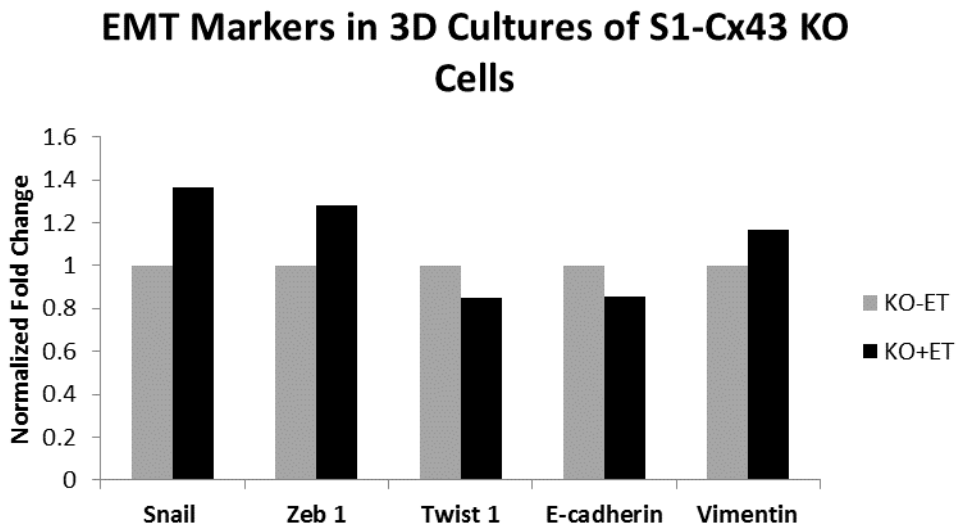
CHAPTER A-II

FIGURES

A



B



Appendix-Figure 1: Real-time PCR using Snail, Zeb-1, Twist-1, E-cadherin and Vimentin forward and reverse primers was performed. Histograms represent the normalized fold change expression levels of the different EMT markers against GAPDH in non-treated and long-term treated (A) 2D cultures of S1 cells and (B) 3D cultures of S1-Cx43 KO S1 cells.

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