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Examining the Functional Consequences of Tumor Endothelial Marker 8 (TEM8) Expression in Human Breast Cancer Cells

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Examining the Functional Consequences of Tumor Endothelial Marker 8 (TEM8) Expression in Human Breast Cancer Cells

by

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Abstract

Tumor Endothelial Marker 8 (TEM8) is a type-1 transmembrane protein overexpressed in tumor associated endothelial cells. Preliminary data from our lab found markedly elevated levels of TEM8 in invasive human breast cancer cell lines of the basal subtype, and reduced TEM8 expression in non-invasive breast cancer cells of the luminal subtype. The functional significance of TEM8 expression in a human breast cancer context is unknown. This thesis explored the consequences of overexpression of TEM8 in non-invasive breast cancer cell lines. MCF7, HTB20, and SKBR3 cells constitutively express low levels of TEM8. The cell lines were infected with lentivirus encoding pLentiTEM8 or control pLentiLacZ. Functional alterations in tumor cell behavior (i.e. viability, apoptosis, adhesion, migration, and invasion) were investigated. NOD-SCID mice were used to evaluate changes in tumor kinetics. In silico data provided by MediSapiens™ was used to assess the prognostic significance of TEM8. The results of this thesis revealed that TEM8 is not sufficient to alter the behavior of non-invasive breast cancer cells to a more malignant phenotype. In vivo studies revealed overexpression of TEM8 does not enhance tumor growth in female NOD-SCID mice. Knocking out host derived TEM8 in MMTV-neu mice had no impact on tumor growth and mortality. In silico data delineate a relationship between TEM8 expression and loco-regional lymphatic spread. The significance of this thesis is that TEM8 alone is not sufficient to cause alterations in the biological behavior of MCF7, HTB20, and SKBR3; however, it may predict loco-regional spread of breast cancer to lymph nodes.

Acknowledgements

Thanks to Oliver Bathe for the opportunity, and my parents for un-flinching support. I need to thank Michael Opoku-Darko, Carol Yuen, and Kathy Gratton for preliminary TEM8 work that paved the way. Michelle Dean was instrumental in carrying out the mouse knockout studies. I would also like to thank my co-supervisor Steve Robbins for stimulating ideas. Thank you to Gwyn Bebb and Don Fujita for continued support and encouragement through thick and thin. This thesis would not have been possible without funding from the CIHR-TRTC and ACF.

Dedication

This thesis is dedicated to Paul and Debra Sampson.

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List of Symbols, Abbreviations and Nomenclature

aa	Amino acid
Ab	Antibody
Ad	Adenovirus
AJCC	American Joint Committee on Cancer
BSA	Bovine serum albumin
C1	Collagen-1
CAM	Cell adhesion molecule
CD	Cluster of differentiation
CMG2	Capillary morphogenesis protein - 2
Col IV	Collagen-4
CSC	Cancer Stem Cell
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
ECM	Extracellular Matrix
EMT	Epithelial to mesenchymal transition
Fc	Fragment crystallisable region (of antibody)
FITC	Fluorescein isothiacyanate
FN	Fibronectin
GFP	Green fluorescent protein
H&E	Haematoxylin and Eosin
HBSS	Hank Balanced Salt Solution

HUVECs	Human umbilical vein endothelial cells
IF	Immunofluorescence
IHC	Immunohistochemistry
KO	Knock Out
L1	Laminin-1
LacZ	Beta-galactosidase gene
Lenti	Lentivirus
LN	Lymph node
MCF	Michigan Cancer Foundation
MMTV	Mouse mammary tumor virus
mRNA	Messenger ribonucleic acid
OE	Over expression
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
qRT-PCR	Quantitative real time polymerase chain reaction
RIP-Tag	Rat insulin promoter SV40 large T antigen transgene
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
TEM8	Tumor Endothelial Marker 8
TEM8-Fc	TEM8 extracellular domain linked to Fc portion of IgG1

TNBC	Triple negative breast cancer
TNM	Tumor, Lymph Nodes, Metastasis
U of C	University of Calgary
VEGF	Vascular endothelial growth factor

Epigraph

"Would you like me to give you a formula for success? It's quite simple, really. Double your rate of failure. You are thinking of failure as the enemy of success. But it isn't at all. You can be discouraged by failure or you can learn from it, so go ahead and make mistakes. Make all you can. Because remember, that's where you will find success."

Thomas John Watson, Sr.
February 17, 1874 – June 19, 1956
Former Chairman and CEO of IBM

CHAPTER ONE: INTRODUCTION

1.1 Breast Cancer: The Clinical Problem

1.1.1 Breast Cancer Epidemiology

Breast cancer is the most common malignancy affecting women worldwide. Overall, breast is the second most frequent cancer site, second only to lung [1]. Studies examining global breast cancer incidence and mortality indicate that in 2008, 1.4 million cases were diagnosed which represents 23% of all female cancers [2]. Geographically, breast cancer frequency is unevenly distributed with the highest incidence in the industrialized regions of Europe and North America [3]. Breast cancer is the most frequent cause of cancer death among females, with an estimated 460,000 annual deaths representing 14% of all female cancer related deaths [2].

When looking at breast cancer from the Canadian perspective it ranks first in terms of incidence among females, and second in mortality with 5-year survival rates of 88% for 2013 [4]. The majority of deaths from breast cancer result from dissemination of primary tumor cells to distant sites. Metastasis is responsible for approximately 90% of deaths from all cancer types, including breast [5].

1.1.2 Metastatic Disease in Breast Cancer

At the time of initial diagnosis approximately 6% of breast cancer patients present with distant metastatic disease [6]. Interestingly, at 3 years, 10-15% of patients diagnosed with local disease will have advanced disease with distant metastasis [7]. Even with improved treatment modalities it is estimated that between 20-30% of those diagnosed

with early stage breast cancer will eventually experience relapse with distant metastatic disease [8]. Most importantly, distant metastasis is associated with a significant decrease in survival at 5 years with rates rarely exceeding 20% [9], compared with 74%-88% for early stage breast cancers [10]. Median survival for women with metastatic breast cancer is 18-24 months [11].

1.2 The Metastatic Cascade as it Pertains to Breast Cancer

The development of metastasis in breast cancer is a critical issue that requires extensive study. By understanding the processes, factors, and mechanisms responsible for the development of metastasis we may be able to greatly improve patient outcomes. Clinically it has become evident that even early breast cancers (small size with no lymph node involvement) have frequently metastasized. The observation that systemic chemotherapy improves outcomes in patients with early breast cancer supports this declaration [12, 13]. Accordingly, identifying molecular events that promote metastasis are critical for the development of novel therapeutics.

Breast cancer has a predictive pattern of metastasis in advanced stage disease. Large scale retrospective post mortem autopsy studies have consistently shown that the primary sites of breast cancer metastasis are the lung, bone, liver, and brain [14, 15]. Additionally, Disibio et al. found the metastatic burden in breast cancer to be hefty, with a tendency toward widespread metastases (5.2 metastases/primary tumor). These observations may explain the continuing difficulties in treating metastatic breast cancer [15].

1.2.1 Prevailing Model of Metastasis

Metastasis is a complex multistep process in which the primary tumor undergoes a variety of biological changes which promote the spread of disease to distant organs. Formation of the primary *in situ* tumor occurs with an intact basement membrane. The primary breast tumor cells undergo alterations in cell to cell adhesion due to a reduction in expression of epithelial cell adhesion proteins such as E-Cadherin [16]. Subsequent up-regulation of adhesion proteins, such as N-Cadherin, allow for adhesion of breast cancer cells to stromal cells thereby facilitating movement through the ECM by virtue of heterotypic cellular interactions [17]. The adherence of tumor cells to the ECM is also mediated by integrins which allow binding of cells to fibronectin, collagen, fibrinogen, and laminin, all components of the ECM. Invasion through the ECM is preceded by degradation of the ECM by up-regulation of MMPs at the invasive front of the tumor. Tumor cells undergo epithelial to mesenchymal transition which assists in tumor cell migration and invasion through the degraded ECM into the circulatory system via haematogenous and or lymphatic routes [18, 19]. Cells that have undergone EMT have an elongated fibroblast like shape and their movement is facilitated by the ECM channels produced by degradative MMPs. After entering the circulation these tumor cells travel through the vasculature, evade the immune system, and arrest in capillaries at sites distant from the primary tumor [20-22]. CAMs and other cell surface adhesion proteins allow for cellular arrest on endothelial cells [23]. Subsequent steps involve extravasation of tumor cells through the endothelium and basement membrane, followed by invasion and migration through the ECM to the secondary site. Once at the secondary site these

aggressive tumor cells *may* seed the target organ and proliferate to form a microscopic growth that *may* eventually form a macroscopic secondary tumor [24] (Figure 1).

Understanding the biology and cellular changes taking place that promote malignant transformation of breast cancer cells will make it possible to perhaps prevent these changes thereby reducing the potential for a metastatic event to occur. Interfering with tumor cell viability, apoptosis, adhesion, migration and invasion could potentially influence the metastatic propensity of breast cancer cells.

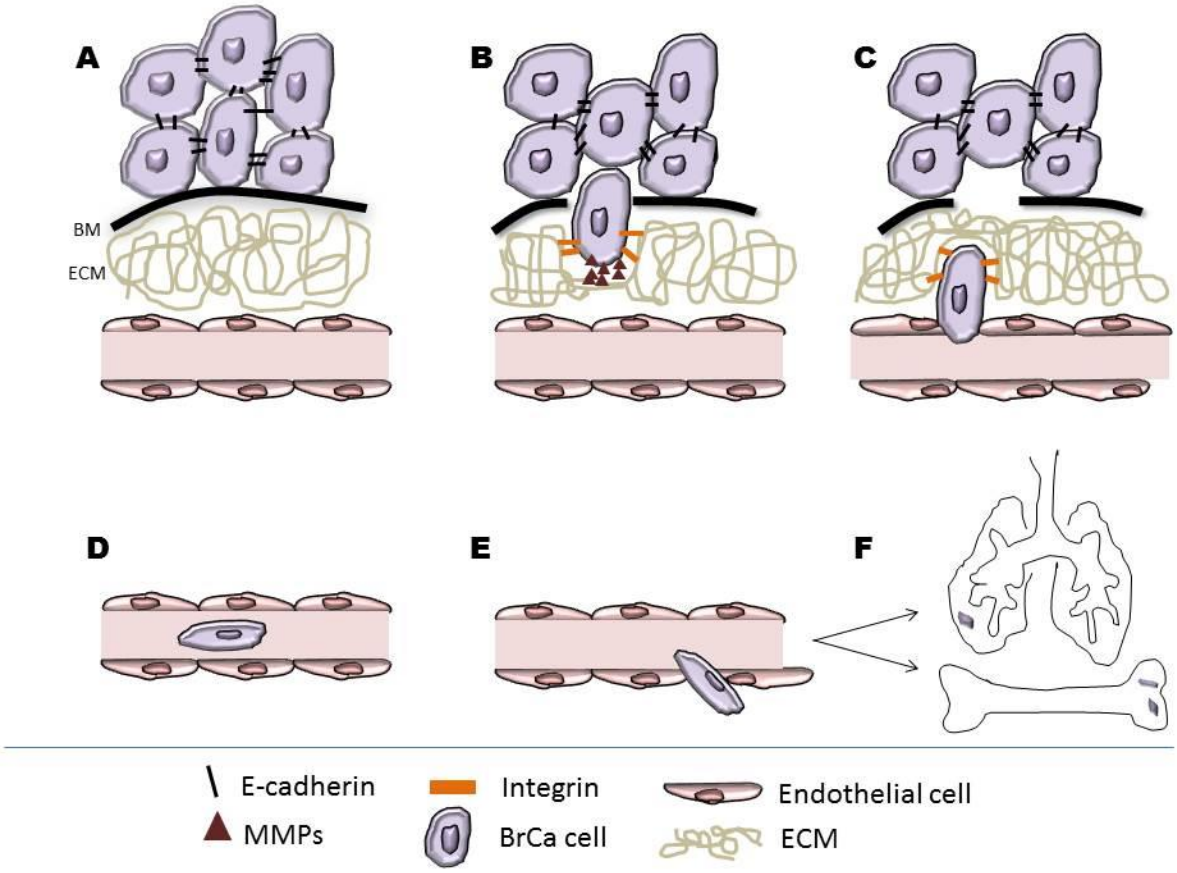

Figure 1 | The Metastatic Cascade

(A) in situ tumor cells surrounded by intact basement membrane (BM). (**B)** Invasion through the BM and beginning of migration through the extracellular matrix. (**C & D)** Metastasizing cells directly enter the circulation through endothelial cells. (**E)** Survival and arrest of tumour cells on endothelial cells followed by extravasation. (**F)** Metastatic colonization of the distant site. Upon colonization of the distant site progressively growing, angiogenic metastases may form.

1.2.2 Seed and the Soil

In the context of breast cancer there appear to be organ sites where tumor cells have a greater propensity toward colonization such as the bone, liver, and brain [14, 15]. The English surgeon Steven Paget recognized this in 1889 when he suggested that the sites affected by metastasis do not appear to be random. He hypothesized that cancer cells (the seed) merely represent one part of the process and they must find the right environment (soil) to help promote growth and the formation of a metastatic growth [25].

Over a century has passed since this observation and a wealth of knowledge has been acquired that relates to both the "seed" and the "soil." Delineating tumor cell and micro environmental interactions which promote tumor growth and metastasis have helped to identify a variety of factors involved in tumor growth and metastasis. Breast cancer cell subsets with a predilection toward specific organ sites, such as the bone and lung, have distinctive molecular signatures that predict metastasis to these sites [26-28]. This suggests certain breast tumor cells have tissue tropism and home to a specific secondary site. An appropriate microenvironment at these distant sites is likely required for breast cancer cells to establish tumor growth and metastatic colonization.

1.2.3 Breast Tumor Microenvironment & Metastasis

Historically breast tumors were thought to be a homogenous population of rapidly proliferating cells that acquired molecular changes over time. This certainly is not the case. The tumor microenvironment is a complex milieu of various cellular elements,

matrix proteins, blood vessels and inflammatory mediators. Studies have begun to elucidate the complex nature of the tumor microenvironment and its influence on tumor biology[29]. The tumor microenvironment contains heterogeneous sub-populations of cancer cells with enhanced malignant phenotypes associated with aberrant proliferation, apoptosis, adhesion, migration and invasion [30, 31]. There are tumor associated endothelial cells with distinctive gene expression profiles that promote tumor angiogenesis [10, 32]. Tumor vasculature tends to be distorted, resulting in hypoxia. In response to hypoxic stress, breast tumor cells release VEGF which increases the abnormal vasculature even more [33, 34]. VEGF expression, microvessel density and angiogenesis are correlated with worse prognosis [35, 36].

A crucial cellular component of the tumor microenvironment includes immune inflammatory cells. Immune cells can be tumor antagonizing (CTLs & NKs) which is expected. Evidence suggests that there are a wide array of immune effectors, particularly macrophages, which interact with breast tumor cells to promote tumor growth and metastasis [37, 38]. Other cell types include tumor associated fibroblasts also found in the tumor microenvironment. Fibroblasts are responsible for creating the structural foundation that supports epithelial tissues by secreting components of the extracellular matrix. Within the tumor microenvironment it is likely that alterations in the ECM, due in part to cancer associated fibroblasts, are responsible for modifications in breast tumor cell biology [39, 40]. The interactions between cancer cells, endothelial cells, immune effector cells, and fibroblasts in the tumor microenvironment are quite well studied. Conversely, interactions between cancer cells and the ECM of the tumor

microenvironment are not very well understood. It is imperative that we identify breast cancer cell molecules that allow interaction between breast tumor cells and the ECM of the tumor microenvironment. Breast tumor cell-ECM interactions likely play an important role in cellular growth, apoptosis, adhesion, migration, and invasion. One molecule that may be involved in breast tumor cell – ECM interactions is Tumor Endothelial Marker 8.

1.3 Tumor Endothelial Marker 8 (TEM8)

1.3.1 Structure

A factor that may be involved in the metastatic cascade, and more specifically breast cancer-ECM interaction, is Tumor Endothelial Marker 8 (TEM8). TEM8 was initially discovered by serial analysis of gene expression (SAGE), and its expression was increased in endothelial cells derived from malignant colorectal tissue [32]. Tumor Endothelial Marker 8 (TEM8) is a type-1 transmembrane protein. The TEM8 gene is expressed as three alternatively spliced mRNA transcript variants that all share an identical extracellular domain[41]. TEM8.1 and TEM8.2 are both membrane bound with TEM8.2 having a shortened cytoplasmic tail, while TEM8.3 is a secreted form of the protein (Figure 2). TEM8.1 contains a single membrane spanning domain, and a 220 amino acid intracellular domain that does not contain consensus sequences for any known structural or functional polypeptides[42]. The TEM8 extracellular domain contains a region that is highly related to von Willebrand factor type A domains. This domain is

known to be involved in protein interactions with alpha integrins and many extracellular matrix components [43] hinting TEM8 may play a role in adhesive functions.

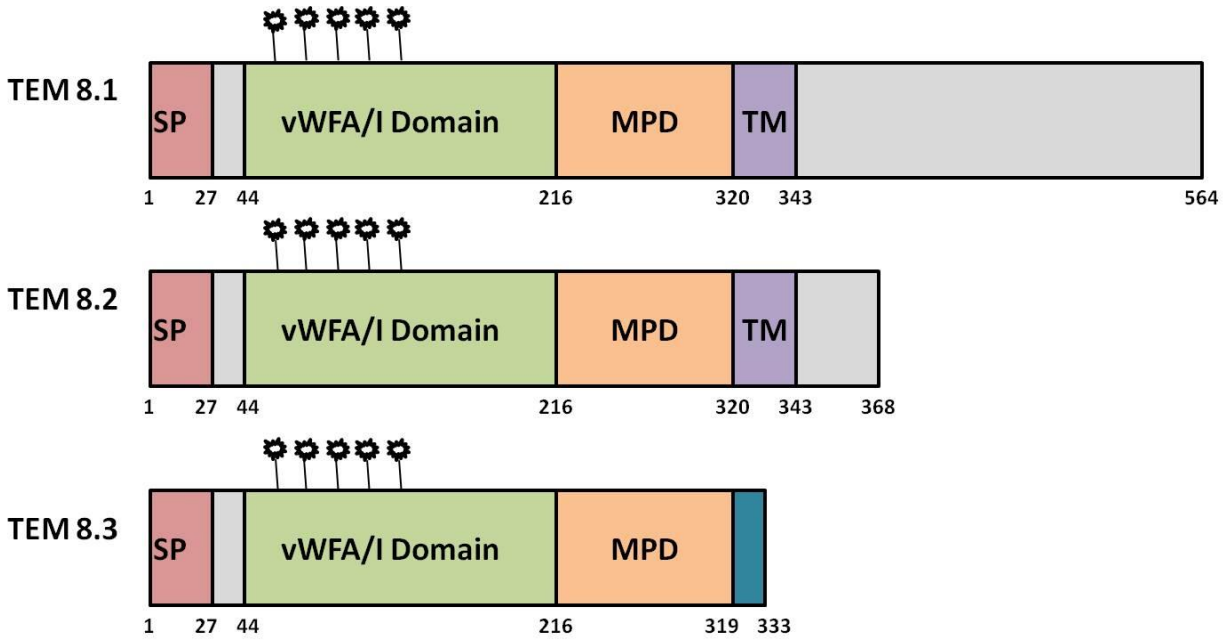

Figure 2 | Schematic of Tumor Endothelial Marker 8 (TEM8)

TEM8 is a type 1 transmembrane protein that has three alternatively spliced transcript variants producing three isoforms of the protein. TEM8.1 and 8.2 are membrane bound, while TEM 8.3 is a secreted version of the protein. SP-signal peptide; vWFA/I Domain-Von Willebrand Factor A / Integrin Domain; MPD-Membrane Proximal Domain; TM-Transmembrane Domain; MIDAS-Metal Ion Dependent Adhesion Sites (black lines with head within vWFA domain)

1.3.2 TEM8 Function

TEM8 is hypothesized to be important in tumor specific angiogenesis, which is a crucial component of metastasis [32, 44]. TEM8 has been shown to be differentially expressed in breast cancer endothelial cells. It is not expressed in the corpus luteum and the granulation tissue of healing wounds which reduce the chances of unintended adverse effects if targeted therapeutically [45]. Moreover, TEM8.1 and 8.2 are localized to the cell surface, further enhancing therapeutic targeting. Murine TEM8 is 96% homologous with human TEM8 and is conserved in murine tumors, so it is likely that murine TEM8 represents an orthologue to human TEM8 so studies of TEM8-directed therapeutics in mice are relevant to humans [45].

Recent studies indicate that up regulation of TEM8 may not be tumor endothelium specific. TEM8 up regulation in breast cancer, at both the transcriptional and protein levels, is associated with an increase in nodal involvement and disease progression [46, 47]. Immunohistochemical analysis of triple negative breast cancer (PR-, ER-, Her2-) shows enhanced TEM8 in cancerous breast tissue compared to non-cancerous breast tissue. TEM8 was localized to the tumor stroma, with focal immunoreactive areas within the tumor[48]. TEM8 was not expressed in normal lymphoid tissue, but showed expression at sites of LN metastases [49] implying TEM8 may be involved in loco-regional lymphatic spread.

The association between increased TEM8 expression and aggressive tumor biology, particularly in breast cancer and colon cancer, has been well defined from the clinical

perspective [46, 47, 50, 51]. Furthermore, published data suggest a role for TEM8 in predicting recurrence of breast cancer after adjuvant CMF-based chemotherapy [52]. To date there are very few data relating variable TEM8 expression to functional alterations in tumor cell behavior. Werner *et al.* have suggested TEM8 is a new adhesion molecule linking collagen-I to the actin cytoskeleton, and thereby plays a supportive role in cell spreading [53]. TEM8 has been found to interact with the ECM proteins collagen-I and collagen-VI as well [54].

$TEM8^{-/-}$ mice are viable. Interestingly, histopathologic analysis reveals an excess of extracellular matrix in several tissues, including the ovaries, uterus, skin, and periodontal ligament of the incisors. These data link TEM8 with ECM homeostasis [49]. When challenged with B16 melanoma, tumor growth was delayed in $TEM8^{-/-}$ mice, the growth of other tumors, such as Lewis lung carcinoma, was unaltered. These studies show that host-derived TEM8 promotes the growth of certain tumors [49].

1.4 Preliminary Data

Experiments were undertaken by Kathy Gratton and Michael Opoku-Darko of the Bathe Lab to look at the expression of TEM8 in a panel of human breast cancer cells. The effect of overexpression of TEM8 on murine 4T1 mouse mammary carcinoma in vivo was also assayed.

1.4.1 TEM8 Expression in Human Breast Cancer Cell Lines

Transcription of each TEM8 isoform was assessed in eight breast cancer cell lines using qRT-PCR. Importantly, there was a dichotomy in TEM8.1 and TEM8.3 expression. Hs578T, MDA MB 468, MDA MB 436, and MDA MB 231 expressed high levels of TEM8 while MCF7, HTB-20, and SKBR3 breast cancer cell lines expressed little or no TEM8. Analysis of the characteristics of the cell lines indicate high TEM8 expressers are typified by the more aggressive basal subtype, and the low expressers the less aggressive luminal subtype of breast cancer [55].

1.4.2 Effect of TEM8 on tumor progression and metastasis in vivo

The effect of TEM8 on tumor biology was elucidated utilizing a murine 4T1 breast cancer mouse model. 4T1 mouse mammary carcinoma cells transduced with recombinant adenovirus encoding AdTEM8.1-GFP, AdTEM8.3-GFP, or Ad GFP were injected into female Balb/C mice (n=7/group). Mice inoculated with 4T1/AdTEM8.1 GFP had increased tumor growth when compared with control 4T1/AdGFP mice. Mice injected with 4T1/AdTEM8.1 GFP also had significantly more lung and lymph node metastases. These data divulge a role for TEM8 in augmenting breast tumor growth and metastasis.

1.4.3 Limitation of Observations

The mouse 4T1 tumor kinetic data is limited in that a mouse cell line was used with transient expression of TEM8. By the same token, control mice had an empty GFP vector, and GFP has been found to be toxic to cells by inducing apoptosis [56]. The

mouse model represents an induced tumor model as opposed to a spontaneous tumor model which would better represent human biology. However, with those limitations in mind, the preliminary data indicates TEM8 may alter breast cancer cell metastatic proclivity.

CHAPTER TWO: HYPOTHESIS AND SPECIFIC AIMS

2.1 Hypothesis

Breast cancer cells with elevated TEM8 expression have enhanced metastatic potential. The cause for the enhanced aggressiveness of breast cancer cells expressing high levels of TEM8 is multifactorial. Alterations may occur in breast cancer cell proliferation, apoptosis, adhesion, migration, and invasion.

2.2 Specific Aims

Aim 1: The prognostic significance of TEM8 in breast cancer will be evaluated.

Clinical data from Davies et al. provide evidence that TEM8 expression is associated with truncated disease free survival. Initially TEM8 mRNA levels in LN+'ve and LN-'ve invasive ductal adenocarcinoma cells was assessed using laser capture microdissection (LCM). The technique was very challenging. Experimental difficulties ensued. Alternatively, we used MediSapeins™ IST *in silico* pathology report to look at a variety of clinico-pathologic characteristics related to TEM8 expression in breast cancer.

Aim 2: The in vitro characteristics of breast cancer cells as a function of TEM8 expression will be assessed.

Data relating TEM8 expression to proliferation, apoptosis, adhesion, migration and invasion in a breast cancer cell context are limited. Data from our lab suggest a correlation between TEM8 expression and breast cancer invasiveness [55]. *In vitro* studies with TEM8 overexpressing breast cancer cell lines were used to determine what effect TEM8 has on the biological behavior of breast cancer cells. Human breast cancer

cell lines MCF7, HTB20, and SKBR3 all express low levels of TEM8. These cell lines were infected with recombinant lentivirus encoding TEM8.1 or LacZ. In vitro functional assays were performed.

Aim 3: The in vivo characteristics of breast cancer cells as a function of TEM8 will be assessed.

Experiments in our lab have shown 4T1 cells infected with AdTEM8.1-GFP have more aggressive breast tumor biology compared to control 4T1 AdGFP cells. This experiment was performed with a murine mammary carcinoma cell line, and the cells were not stably infected. An examination of tumor kinetics as a function of TEM8 expression in stably infected HTB20 human breast cancer cells was used to examine the effect of TEM8 on tumor growth *in vivo*. We also utilized a spontaneous MMTV-neu mouse tumor model comparing $TEM8^{+/+}$, $TEM8^{+/-}$, and $TEM8^{-/-}$ mice. The spontaneous MMTV-neu tumor model more closely resembles the human situation.

CHAPTER THREE: MATERIALS & METHODS

3.1 Cell Lines

SKBR3 and HTB20 cell lines were maintained in high glucose 1X Dulbecco's Modified Eagle Medium (DMEM) from GIBCO supplemented with 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37℃ in a humidified atmosphere of 5% CO_2 conserved in a ThermoScientific HeraCell 150i incubator. MCF7 cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 0.01mg/mL insulin, 1X Non Essential Amino Acids (NEAA), and 1X Sodium Pyruvate.

The corresponding lentirviral breast cancer cell constructs (described below) were grown in complete 1X DMEM supplemented with 5 µg/mL blasticidin for SKBR3 and HTB20 and 8µg/mL blasticidin for MCF7 cell lines. At least 24 hours prior to all described assays, cells were withdrawn from blasticidin supplemented DMEM and supplied with blasticidin free DMEM. *All assays were carried out in blasticidin free media*.

3.2 Cell Harvesting: Trypsin vs. Puck's/EDTA

0.25% Trypsin-EDTA was initially used to harvest cells, but proteolytically cleaved TEM8 and other cell surface proteins. A non enzymatic cell dissociation reagent Puck's/EDTA was used to harvest cells. Puck's EDTA contains 4mM, NaHCO3; 136mM, NaCl; 4mM, KCl; 1mM, EDTA; 1mg/ml dextrose; 10mM, HEPES. The solution was mixed, pH 7.3-7.4, then filtered at 0.2µm. Cell scraping with Puck's/EDTA was the preferred method of harvesting cells for all described assays so as to minimize TEM8 cleavage.

3.3 Gateway Lentiviral Constructs

Gateway Lentiviral constructs were made by Kathy Gratton. Kathy used The Invitrogen™ pLenti6.3/V5-DEST Gateway Vector Kit (catolog no. V533-06) to construct breast cancer cell lines overexpressing TEM8 as per the manufacturer's instructions [57]. MCF7, HTB20, and SKBR3 cell lines were all stably infected with pLentiTEM8 or control pLentiLacZ.

3.4 Western Blots

Cells were lysed in NP40 lysis buffer (NP40, 1% PMF, 1% NaOV, 0.1% protease inhibitor), insoluble materials were pelleted, and the concentration of protein within the supernatant was determined using the BioRad D_C Protein Assay. Equal amounts (15-25ug) and volumes of protein samples were loaded into and run on a 10% polyacrylamide gel. Proteins were separated, and blotted onto 0.45µm pure nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% milk in TBST (20mM Tris-HCL, 150mM NaCl, 0.1% Tween-20) to reduce non-specific antibody binding. The blot was incubated overnight at 4°C with SB5, a monoclonal mouse anti-human TEM8 antibody provided by Brad St. Croix. The membrane was incubated with an HRP-linked goat-anti-mouse IgG secondary antibody. Specific labeling was observed using Western Lightning™ Enhanced Chemiluminescent (ECL) reagents. Blots were imaged using Kodak X-OMAT 2000 processor. Blots were finally stripped and probed for β-actin (Santa Cruz, sc-1616, goat polyclonal IgG) as a loading control.

3.5 MTT Cellular Viability Assay on ECM Substrates

The wells of a Corning flat bottom 96 well plate were coated with 0.1% BSA, 5ug/cm^2 collagen-I (BD Biosciences, cat no. 354236), 5ug/cm^2 Fibronectin (BD Biosciences, cat no. 354008) or with Matrigel (BD Biosciences, cat no. 356237) diluted 1:50 (~0.3mg/mL). Cells were harvested, and plated in the pre coated 96 well plate at a pre-determined optimal density of 1.25 x 10^4 cells. The plate was incubated in a humidified chamber at 37^{o}C and 5% CO_2 for between 48 and 72 hours. At the experimental endpoint 5mg/mL MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma, cat no. M2128-5G) was added at a volume of 10uL per 100uL media. Cells were treated with MTT for 4 hours. Purple formazan was solubilised with 100µL sterile Dimethyl sulfoxide (DMSO) on an orbital shaker in the dark for 30 minutes. The plate was read on BioRad Benchmark Plus microplate spectrophotometer at 590nm, with 620nm background subtracted ($A_{590} - A_{620}$).

3.6 Cell Adhesion

The CytoSelect^{TM} 48 Well Cell Adhesion ECM Array Colorimetric Format (Cell Biolabs, Inc., cat no. CBA-070) assay was used to evaluate cellular adhesion to Fibronectin, Collagen I, Collagen IV, Laminin I, and Fibrinogen. Under sterile conditions the ECM Adhesion plate was warmed to room temperature. Cell suspensions were prepared containing 1.0 x 10^6 cells/mL in serum free DMEM containing 0.5% Bovine Serum Albumin (BSA), 2mM $CaCl_2$, 2mM $MgCl_2$. 150µL of the cell suspension was added to the inside of each well, placed in a cell culture incubator for *90 minutes*. Media was carefully discarded from each well, and gently washed 4 times with 250uL 1X PBS

containing 2mM $CaCl_2$ and 2mM $MgCl_2$. 200µL of Cell Stain Solution was added to each well for 10 minutes, discarded, and the wells were gently washed 5 times with 500µL deionized water. Wells were air dried and then 200µL extraction solution was added per well and placed on an orbital shaker for 10 minutes. 150µL from each extracted sample was added to a 96 well microtiter plate, and the optical density (OD) was measured at 560nm with the BioRad Benchmark Plus microplate spectrophotometer.

3.7 Cell Migration

The migratory ability of cells on tissue culture plastic, fibronectin, and collagen-I was assayed using the Oris™ TriCoated Cell Migration Assay (Platypus Technologies LLC, cat no. CMATR1). The assay utilizes a 96 well plate, and cell seeding stoppers made from silicone to restrict cell seeding to the outer regions of the wells. Removal of the stoppers reveals the 2mm unseeded region in the center of each well, into which the seeded cells may migrate. A detection mask is applied to the bottom of the plate to restrict visualization of fluorescently labelled migratory cells to the 2mm detection zone (Figure 3).

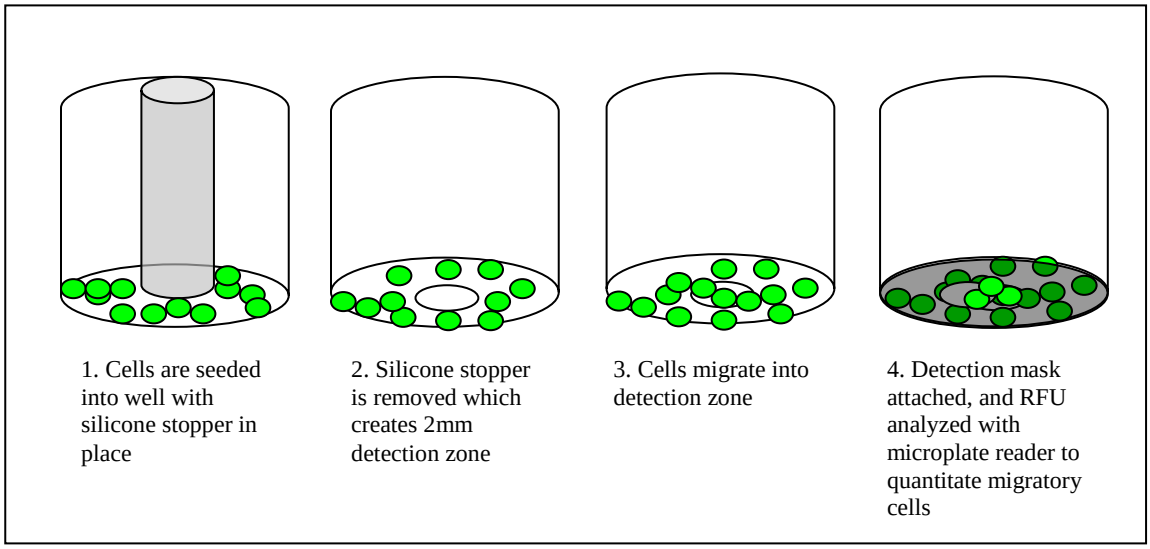

Figure 3 | Schematic of Cell Migration Assay

The underside of the 96 well plate was inspected to ensure the silicone cell seeding stoppers were firmly sealed against the bottom of the plate. Cells were harvested and prepared at a dilution of 2.5 x 10^5 cells/mL and 100µL of suspended cells were added into each test well through the side ports of the cell seeding stoppers. The plate was incubated in a humidified chamber (37^{o}C, 5% CO_2) for 8 hours to permit cell attachment. Stoppers were then removed, except from the reference wells for each surface treatment (tissue culture, fibronectin, collagen-1) in which the stoppers remain in place until the results are obtained thereby representing t=0h migratory controls. After stoppers were removed, wells were gently washed with 100uL 10% DMEM to remove unattached cells. 100uL of fresh culture media was added to each well, and the plate incubated for 16h in a cell culture incubator. After 16h 5uL of 1mg/mL Calcein AM (Invitrogen, cat no. C3100MP) in DMSO was added to 10mL serum-free media. Culture medium was carefully discarded from wells, and the wells were washed. 100uL of diluted Calcein

AM solution was added to each well, and the plate was incubated at 37^{o}C for 30 minutes. Lastly, the detection mask was attached to the plate, and the plate was analyzed using Molecular Devices SpectraMax M2 microplate reader with 494/517nm excitation/emission filters and high photomultiplier tube (PMT) sensitivity.

3.8 3D Culture Invasion Assays

The ORIS cell invasion assay is a 3-D assay for investigating cell invasion of adherent cell lines. Cells were serum starved for 24h prior to experiment in 0.5% FBS. 100µL of 3.5mg/mL Basement Membrane Extract (BME) coating solution was used to coat all wells of the 96 well plate. The plate was populated with Oris cell seeding stoppers. Cells were harvested using non-enzymatic cell disassociation reagent at 2.5 x 10^5 cells/mL. 100µL of cells/well were seeded, except for t=0 pre-invasion reference wells which were seeded to 75% confluence. The seeded plate with cell seeding stoppers was incubated at 37^{o}C, 5% CO_2 for 8 hours to allow attachment. Cell stoppers were removed, media was removed, and wells were gently washed with serum free media to remove unattached cells. 40µL of the BME stock reagent was added to each well to create at 3-D BME overlay. The plate was incubated in a humidified chamber at 37^{o}C, 5% CO_2 for 30 minutes to permit polymerization of the 3D BME overlay. 100µL of cell culture medium ± FBS was added on top of the BME overlay. Plate was incubated for t=48h. 100uL of diluted Calcein AM solution was added to each well, and the plate was incubated at 37^{o}C for 30 minutes. Detection mask was attached to the 96 well plate, and the plate was analyzed using Molecular Devices SpectraMax M2 microplate reader with 494/517nm excitation/emission filters and high photomultiplier tube (PMT) sensitivity.

3.9 Apoptosis

Apoptosis was evaluated by fluorescence-activated cell sorting (FACS). 2.0×10^6 cells were plated on a 100 x 20mm tissue culture dish and serum starved for 48h. Cells were subsequently harvested with Puck's/EDTA and cell scraping then centrifuged at 1500rpm for 5min. 1×10^5 cells were transferred to a 5mL polystyrene round bottom FACS tube (Falcon, cat no. 352008) and pelleted. The cell pellet was washed in 1X Phosphate Buffered Saline (PBS), and re-suspended in 500uL 1X Annexin V Binding Buffer. 1uL of Annexin V-FITC reagent (BioVision, cat no. 1001-1000) and 1uL of 250µg/mL Propidium Iodide (PI) (BioVision, cat no. 1056-1) in PBS were added to experimental tubes for which double staining was required. Controls included cells alone, Annexin V-FITC alone, and PI alone. Tubes were incubated at room temperature for 5 minutes in the dark prior to analysis using the BD™ LSR flow cytometer at the U of C Flow Cytometry Core Facility.

3.10 Cell Cycle

Cells were synchronized via serum starvation and then released into 10% DMEM. Cells were harvested at t=0h, t=12h, and t=24h. Harvested cells were washed with ice-cold 1x PBS, harvested with Puck's/EDTA, and used for cell cycle analysis. After collection cells were washed and centrifuged at 1500rpm for 5 minutes. Following another round of centrifugation, the pellet was re-suspended in 2mL of PBS and transferred to a plastic FACS tube. The sample was centrifuged, and the pellet re-suspended in 500µL of PBS and 500µL of ice cold 95% ethanol while gently vortexing. Tubes were stored at 4°C for 24 hours. The next day the cells were centrifuged, washed in PBS, re-centrifuged, and

the supernatant was aspirated. Cell pellet was re-suspended in PI staining buffer (50µg/mL Propidium Iodide / 0.1% Tritonx100 / 0.2mg DNAse free RNAse A in PBS). Cells were incubated in the dark for 45 minutes at room temperature prior to analysis at the University of Calgary Flow Cytometry Core Facility.

3.11 In vivo

In vivo studies were conducted in accordance with the ethical standards of the Animal Care Committee at the University of Calgary, and all studies received ethics approval. Female Non Obese Diabetic/Severe Combined Immunodeficient (NOD/SCID) mice were challenged with either HTB20pLentiTEM8 or control HTB20pLentiLacZ breast cancer cells. Each mouse was challenged with 1.0×10^6 cells in 50µL sterile 1X Hanks Balanced Salt Solution (HBSS) and co-inoculated with 50µL phenol red free BD Biosciences Matrigel. Mice were injected on the right mammary fat pad subcutaneously with a 26 gauge needle, and 1cc syringe. Tumor growth was monitored thrice weekly. The length and width of the tumor were measured with calipers and documented. Tumor volume was calculated using the formula $\frac{1}{2} (L \times W^2)$[58]. Mice were euthanized when tumor dimension measurement exceeded 1.0 cm in either dimension, or if the mouse had deteriorating health.

3.12 Transgenic Mice

MMTV-neu and B6 RIP-Tag TEM8 KO mice were constructed by Michelle Dean of the Bathe Lab. Briefly, MMTV-neu mice which develop spontaneous mammary carcinoma

were bred onto a C57/BL6 (B6) background and crossed with B6 TEM8$^{-/-}$ mice provided by Brad St Croix[49]. Mice were screened for TEM8 genotype by PCR using genomic DNA derived from mouse ears. Tumor burden was monitored weekly. Tumor size >1cm in any dimension was considered experimental endpoint.

B6 RIP-Tag mice were crossed with B6 TEM8$^{-/-}$ mouse provided by Brad St. Croix. TEM8 genotype was evaluated by PCR using genomic DNA derived from mouse ears. B6 RIP-Tag mice develop spontaneous β cell tumors (insulinomas) since they carry the SV40 large T antigen proto-oncogene under the control of the rat insulin promoter[59]. Tumor burden was measured by time to hypoglycemia. Blood glucose levels were measured 2-3 times weekly using a Fasttake glucose monitor (Johnson and Johnson, Burnaby, BC). The presence of insulinoma was marked by two readings ≤ 4 mmol/L (hypoglycemia).

3.13 In Silico Transcriptomics

TEM8 expression data in a human tissues were provided by MediSapeins In Silico Transcriptomics (IST) in the form of an in silico pathology report. The normalized TEM8 expression data from 9,783 human tissue samples were analyzed [60]. We specifically looked at expression of TEM8 in breast cancer and its relationship with various clinico-pathological features. TNM stage, grade, site-specific metastasis, and overall survival were all explored. TEM8 expression in other tumors of epithelial origin

including colorectal carcinoma, lung adenocarcinoma, and gastric adenocarcinoma was also looked into.

3.14 Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). Deviations from normal distribution were tested using the Kolmogorov-Smirnov (KS) test. The statistical significance of the differences between two means from parametric data was tested by the two-tailed Student t test for two independent samples. One-way or two-way ANOVA was used to check for significance depending on the number of variables being evaluated. Bonferonni post hoc test was used to identify significance between subgroups.

Statistical differences between clinic-pathological features and TEM8 expression were mostly evaluated using non-parametric tests. The Mann-Whitney U test was used between 2 groups and the Kruskall-Wallis test was used between 3 or more groups with Dunns multiple comparison post-test if significance between means was observed. Survival analysis was evaluated using Kaplan-Meier analysis, and the the log rank test was used to test for differences in survival between groups. $P<0.05$ was considered to indicate a statistically significant difference. All calculations were performed using GraphPad Prism 5.0 software.

CHAPTER FOUR: TEM8 EXPRESSION IN CLINICAL SAMPLES

4.1 In Silico Transcriptomics

4.1.1 Rationale

Clinical data from Davies et al. provide evidence that TEM8 expression is associated with truncated disease free survival and LN metastasis [47]. Our previous studies have shown that in murine 4T1 cells TEM8 expression promotes tumor growth and metastasis to lymph node and lung [55]. In order to examine the relationship between TEM8 expression and the clinico-pathological features of breast cancer, clinical specimens from MediSapeins™ in silico transcriptomics were utilized.

4.1.2 Introduction

Utilizing MediSapiens™ IST, a comprehensive In Silico Molecular Pathology Report for TEM8 was generated across all breast cancer specimens available. The source of data have been extensively used in peer reviewed research publications [60-62]. The data were used to evaluate a variety of clinical characteristics with respect to TEM8. Breast cancer cell subtype, TNM staging, tumor grade, site specific metastasis, and overall survival were evaluated as a function of TEM8 expression.

4.1.3 Results

4.1.3.1 TEM8 Expression in Breast Cancer Cell Subtypes

Breast cancers can be stratified according to their molecular profile into five subtypes: luminal A, luminal B, basal like, HER2 overexpressing and normal breast-like [63, 64]. Molecular profiles can predict prognosis, with the luminal subtypes tending to have a

better prognosis than both basal like and HER 2 overexpressing tumors; which tend to have poor clinical outcomes [64, 65]. Evaluation of TEM8 in these various breast cancer cell subtypes suggest that there is no statistical difference in TEM8 expression between the groups with $p=0.4886$. Basal cell tumors ($n=110$) had a mean expression value of 372.8 ± 19.01, Her-2 ($n=46$) 408.8 ± 30.65, Luminal-A ($n=117$) 385.1 ± 18.03, Luminal-B ($n=76$) 350.9 ± 20.35, and Normal like ($n=86$) 411.0 ± 25.4. Box and whisker plots showing all characteristics of these data can be seen in Figure 4.

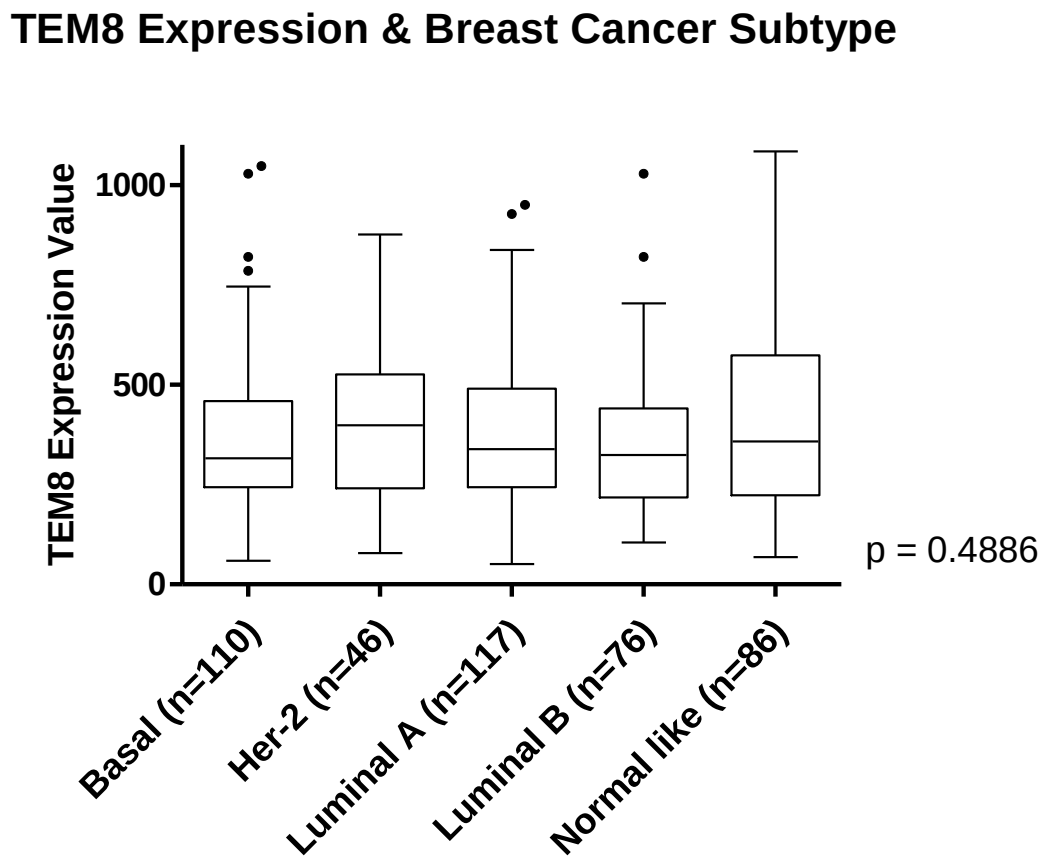

Figure 4 | TEM8 Expression in Breast Cancer Cell Subtypes

Medisapein *in silico* transcriptomic data was used to evaluate TEM8 expression levels in various human breast cancer cell subtypes. There was no statistically significant difference in TEM8 expression with respect to breast cancer cell subtype. Kruskal-Wallis test was used to check for significance and interaction between all the variables tested; breast cancer cell subtype and TEM8 expression value.

4.1.3.2 TEM8 Expression and Disease Stage in Breast Cancer

The TNM staging system was initially devised by Patrick Denoix in 1946 [66]. Since then it is the common method of classification of solid tumors as per the American Joint Committee on Cancer (AJCC) guidelines. T refers to tumor size, N refers to regional lymph node involvement, and M refers to metastatic spread of primary tumor [67]. Histologically tumor grade is typically used in reference to the degree of differentiation of a tumor. Those tumors that appear "normal" microscopically are typically low grade, whereas high grade tumors are poorly differentiated. The *in silico* data gave us the opportunity to look at tumor tissues in the context of these parameters. T-stage, N-stage, M-stage, and tumor grade were all assessed with respect to TEM8 expression at the transcriptional level.

The data indicate no significant difference in TEM8 expression values when observing both tumor size ($p=0.5652$) and distant metastatic spread ($p=0.0957$). Low grade tumors ($n=154$; $\mu=280.3 \pm 10.47$) tended to have lower TEM8 expression when compared to high grade tumors ($n=304$; $\mu=309.4 \pm 7.87$) $p<0.05$. Most interestingly, it appears the $n = 204$ tumor samples with lymph node involvement ($\mu=357.2 \pm 10.59$) have much higher TEM8 expression when compared to the $n = 650$ tumor samples with no nodal involvement ($\mu=281.7 \pm 5.077$) $p<0.001$. Box and whisker plots of all the data are depicted in Figure 5.

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These data infer that high grade tumors may slightly overexpress TEM8. Most importantly the data convincingly reveal a potential role for TEM8 in loco-regional lymph node metastasis. This is a very interesting result as Gutwein et al, have recently demonstrated TEM8 expression in lymph node metastasis from triple negative breast cancer samples [48].

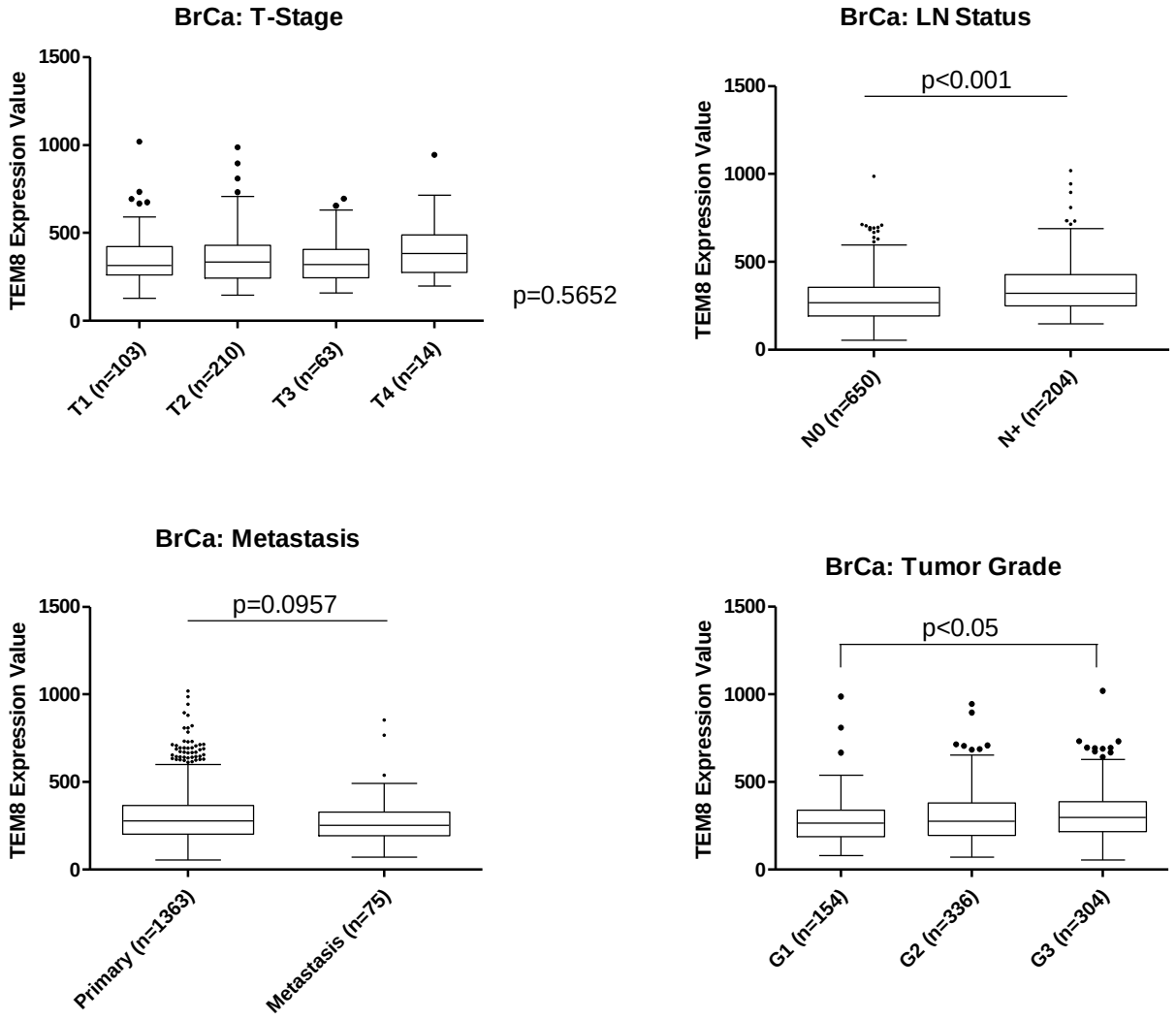

Figure 5 | Relationship Between TEM8 Expression and clinico-pathological features of Breast Cancer

Medisapien in silico transcriptomic data was used to evaluate the relationship between TEM8 expression and various breast cancer features. T Stage, Lymph Node (LN) Status, Metastasis and Tumor Grade were all assessed with respect to TEM8 expression. Note that for T Stage, Lymph Node Status and Tumor Grade the n=75 metastasis samples were excluded. Clinical data was not available for all specimens hence the disparity in sample size. T Stage and Grade were statistically evaluated by Kruskal-Wallis test, followed by Dunns multiple comparison post-test if significance was achieved. α set at $p<0.05$. Lymph Node Status and Metastasis were statistically evaluated using Mann-Whitney U non parametric test.

4.1.3.3 Site Specific Metastasis and TEM8 Expression

Although there was no difference in TEM8 expression between tumor samples that had metastasized and those that did not (Figure 5), there was an opportunity to look at n=75 breast tumor samples that had metastasized to various distant sites. The aim was to observe whether TEM8 is associated with a predilection toward metastasis at a particular distant site. No such data exist relating TEM8 expression to site specific metastasis, although there are a plethora of papers that explore the molecular signature of breast cancer cells that preferentially colonize selected sites [26-28, 68]. Note that TEM8 was unable to be found in any of these gene signatures related to metastatic site specificity.

Interestingly, when TEM8 expression was examined tumor samples that had preferentially spread to bone had higher TEM8 expression when compared with brain, liver, and lung (Figure 6). Bone (n=19) metastasis samples had a μ expression value of 402.6 ± 37.9 compared with 204.6 ± 15.9, 177.1 ± 38.77, and 244.5 ± 23.63 for Brain (n=22), Liver (n=5), and Lung (n=21) respectively. Kruskal Wallis revealed $p<0.0001$, and Dunns multiple comparison post-test indicate that for bone vs brain, lung or liver $p<0.001$. There was no significant difference for bone vs. other. It should be noted that for the "other" category, four samples were from ovary metastasis, one parietal pleura, one small bowel, and one intervertebral disc.

Although the literature has no mention of TEM8 enhancing bone metastasis, the data suggest that tumors that have already metastasized to the bone may have enhanced TEM8 expression when compared to metastatic tumor samples from the Brain, Liver, and Lung.

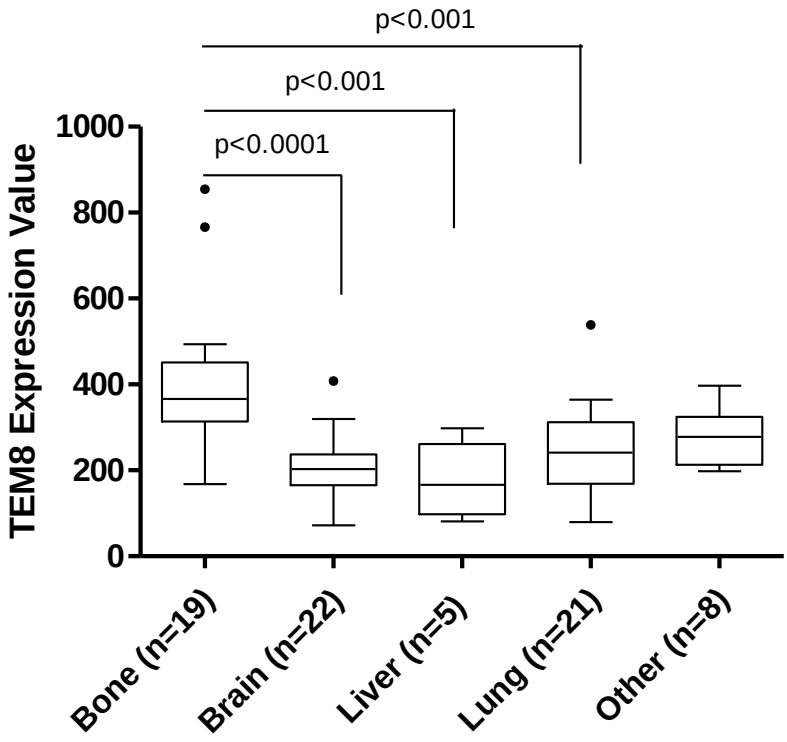

Figure 6 | Expression of TEM8 at site specific metastasis

TEM8 expression in n=75 metastatic samples were assessed as a function of site specificity. There was higher level TEM8 expression in bone samples when compared to brain, liver, or lung. Other Sites included (4 ovary, parietal pleura, small bowel, intervertebral disc). Kruskal-Wallis test was used to check for significance and interaction between TEM8 and site specificity ($p<0.001$). Dunns multiple comparison post-test was used to check for significance with α set at $p<0.05$.

4.1.3.4 Impact of TEM8 on Breast Cancer Overall Survival

The effect of TEM8 on overall survival was appraised. Survival data were available for 767 tissue sample. Median TEM8 expression was 360.7 ± 8.18. Survival curves were generated by taking the bottom 25th percentile (< 249.5 $^{\text{TEM8 Expression Value}}$) of TEM8 expressers and top 75th percentile (> 556.9 $^{\text{TEM8 Expression Value}}$) to create survival curves. Median survival was never reached. It appears that the top 75th percentile of TEM8 expressers had better overall survival than the lower 25th percentile of TEM8 expressers (Figure 7). These data were not statistically significant with $p = 0.0673$ as per Log-rank (Mantel Cox) test. These data do suggest TEM8 does not directly affect overall survival in breast cancer patients, as there are a variety of variables at play.

4.1.3.5 TEM8 and Clinical Correlates in other Adenocarcinomas

We examined TEM8 expression in other cancers of epithelial cell origin to evaluate whether the in silico findings were breast cancer specific or represented a global theme with respect to adenocarcinomas. Specifically we were curios whether the relationship between TEM8 expression and lymph node positivity was universal. Data provided by Medisapiens™ IST allowed evaluation of TEM8 in colorectal cancer (CRC), lung adenocarcinoma, and gastric adenocarcinoma. For CRC, lung adenocarcinoma, and gastric adenocarcinoma there was an increase in TEM8 expression in LN positive samples. The finding was nearly statistically significant in CRC with $p=0.0514$, and $p=0.002$ and $p=0.0166$ for lung and gastric adenocarcinoma respectively by Mann Whitney U test (Appendix 1: Figure 22-24). These findings are very interesting and link TEM8 with lymph node positivity of adenocarcinomas in multiple tissue types.

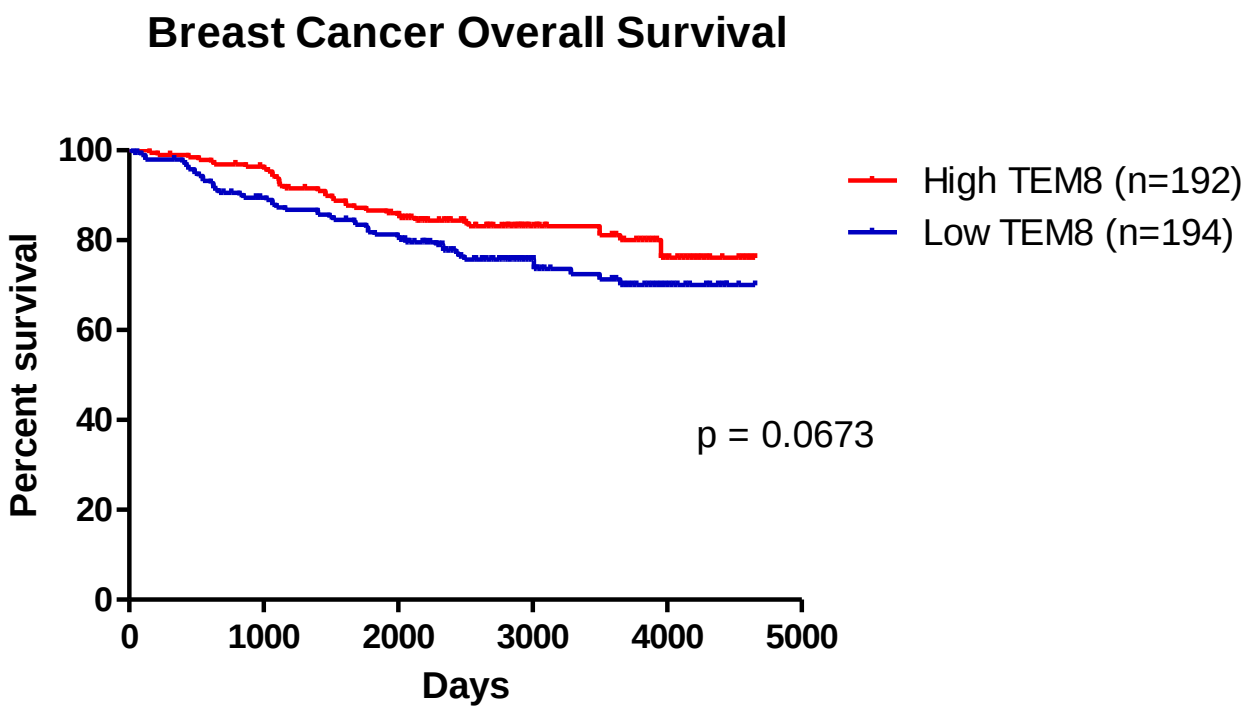

Figure 7 | Effect of TEM8 on Breast Cancer Overall Survival

TEM8 expression was analyzed with respect to overall survival. The bottom 25% TEM8 expressors with a TEM8 expression value less than 249.5, and top 75% TEM8 expressors with a TEM8 expression value greater than 556.9 were grouped to evaluate survival. TEM8 expression did not significantly alter overall survival when the high and low TEM8 expressors were compared. Log-rank (Mantel Cox) test was used to assess significance (p=0.0673).

4.1.4 Chapter Discussion

In silico transcriptomic data was supplied by MediSapiens. Integration of large collections of gene expression data from different tissues and microarray platforms is typically difficult to analyze and compare due to the variety of microarray technologies used. In 2008 Kilpinen et al. developed and validated a novel method to normalize data arising from different Affymetrix microarray generations. Now, 19,000 genes across 20,000 patient samples amongst a variety of normal tissue and cancer tissue types can be compared [60]. A significant proportion of these samples have accompanying data related to prognosis and clinical outcomes.

When looking at breast cancer cell molecular subtypes and TEM8 expression there was disagreement between the MediSapens data and the original qRT-PCR expression data that exposed differential TEM8 expression between basal and luminal breast cancer cell subtypes. Our original data only looked at four "basal-like" cell lines (Hs578T, MDA-MB468, MDA-MB436, MDA-MB231) and three luminal cell lines (MCF7, HTB20, SKBR3). The MediSapiens data looks at TEM8 expression in human tissue which may explain the apparent discrepancy. Additionally, the in silico data are derived from whole tumors, and the results may depict partly TEM8 expression in tumor cells including stromal fibroblasts and endothelial cells.

TEM8 was not associated with differences in T-stage or distant metastasis (M1 disease). This seems counterintuitive since TEM8 plays a well-documented role in angiogenesis and tumor growth [69]. Conceptually, overexpression of TEM8 would enhance tumor

angiogenesis leading to increased tumor growth and an increased probability of haematogenous cancer cell spread. Cautious interpretation of these data is warranted, as these samples are a blend of endothelial, tumor, and stromal elements, and thereby do not represent a homogenous population of tumor cells.

Information related to site specific metastasis was able to be gleaned from MediSapiens data. For the n=75 distant metastasis samples TEM8 expression correlated with distant spread to bone when compared independently to each of Lung, Liver, and Brain. Examining supplementary data from Kang et al., TEM8 is not overexpressed in the transcriptional gene signature for bone metastasis from a single cell progenitor [26]. It is difficult to draw any definitive conclusions with respect to TEM8 and site specific metastasis with data that contravene one another.

Examining the clinical characteristics of breast cancer cells with respect to TEM8 expression revealed that TEM8 could be associated with lymph node metastasis. Previous experiments showed that in a murine 4T1 model, overexpression of TEM8 enhances lymph node metastasis in female Balb/c mice[55]. Davies et al. analyzed mRNA levels via qRT-PCR in a cohort of breast tissue with 6 year follow-up. There data show an association between increased levels of TEM8 with lymph node involvement and disease progression [46]. Interestingly a recent study looking at triple negative breast cancer and expression of TEM8 in human samples revealed that TEM8 is not expressed in normal lymphoid tissue, but shows expression at sites of lymph node metastasis [48]. Triple negative breast cancer does overlap with the molecular entity of basal-like breast

cancer, however they are not entirely the same, and equating them can be misleading [70, 71]. With that caution in mind, there are multiple lines of evidence proposing TEM8 association with lymph node metastasis in breast cancer. Also, we found that in other adenocarcinomas the pattern of association between TEM8 and lymph node positivity existed. Perhaps overexpression of TEM8 promotes loco regional spread of disease via lymphatic routes. This finding is intriguing and needs to be investigated.

When examining survival, high TEM8 expression is not associated with a reduction in survival. There are likely many confounders impacting survival; it is naïve to expect TEM8 expression alone to directly impact breast cancer overall survival. Median survival was never reached in either group for the follow-up period. These are also hard data to interpret. Davies, et al. present data from n=120 tissue samples with median follow-up of 120 months revealing increased TEM8 expression in breast cancer tissues corresponding to shorter median survival (122 vs 134.8 months). The finding was not statistically significant p=0.28 [47].

All together the in silico data reveal that TEM8 may be involved in loco-regional lymphatic spread of primary tumor cells. This finding was consistent in all adenocarcniomas where TEM8 data were available. Similarly, we have previously shown transient expression of murine 4T1 mouse mammary carcinoma cells leads to increased tumor growth and metastasis to lung and lymph nodes. In order to define a specific biological function for TEM8 we sought to functionally characterize the effect of overexpression of TEM8 in human breast cancer cells.

CHAPTER FIVE: FUNCTIONAL EFFECTS OF TEM8 IN VITRO

5.1 In Vitro Studies

5.1.1 Rationale

Initially dichotomous TEM8 expression was observed in luminal and basal breast cancer cell subtypes. Basal breast cancer cell subtypes were found to express higher levels of TEM8 at the mRNA and protein level when compared to luminal subtypes. Experimentally and clinically basal breast cancer cell types tend to behave more aggressively than their luminal counterparts. We wanted to take low TEM8 expressing luminal subtype breast cancer cells and observe whether over expression of TEM8 promoted a more invasive phenotype experimentally.

5.1.2 Introduction

To examine the functional effect(s) of TEM8 on breast cancer cell behavior, overexpression of TEM8 in low TEM8 expressing cell lines was imperative. MCF7, HTB20, and SKBR3 breast cancer cell lines were all low TEM8 expressers at the transcriptional and translational level. These cell lines were used to examine what effect overexpression of TEM8 has on the biological behavior of human breast cancer cells in vitro. All three breast cancer cell lines were used in assays to eliminate cell line dependent findings. In vitro assays were designed to assess the biological characteristics typically associated with invasive cells. The microenvironment was kept as simple as possible to minimize interplay between variables. The microenvironment, in vitro, consisted of ECM substrates in the hope of activating TEM8 to enhance malignant

behavior. Viability ±ECM substrates, cellular adhesion to ECM, resistance to apoptosis, cell cycle analysis, 2D-radial migration, and 3D-cellular invasion were all evaluated.

5.1.2.1 TEM8 Overexpression in Non-Invasive BrCa Cells

The luminal subtype, weakly metastatic, low TEM8 expressing human breast cancer cell lines MCF7, HTB20, and SKBR3 were all infected with recombinant lentiviral vector containing a TEM8 insert. Appropriate expression of TEM8 by these cell lines was established via western blot (Figure 8). Control cell lines were created with an 'empty' LacZ lentiviral vector.

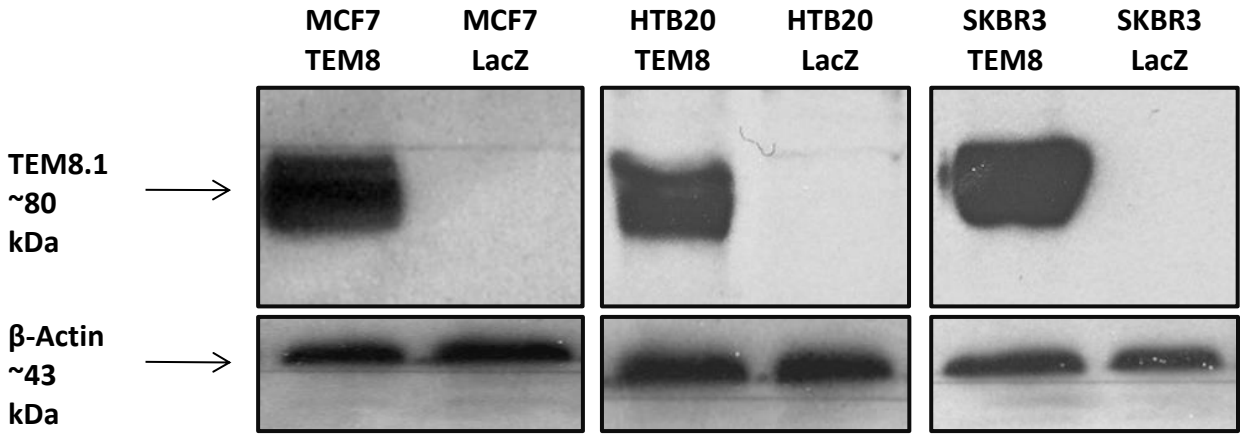

Figure 8 | Overexpression of TEM8.1 isoform in three human breast cancer cell lines

Human breast cancer cells lines MCF7, HTB20 and SKBR3 were infected with TEM8 or control LacZ using the Invitrogen pLenti6.3/V5 DEST lentiviral expression system. Once cells were stably infected with lentiviral vector, western blot was performed to ensure adequate expression of the protein product.

5.1.2.2 Trypsin Cleavage of TEM8

The majority of in vitro experiments initially carried out involved the use of trypsin to harvest adherent cells enzymatically. However, when looking at the bioinformatics data via PeptideCutter, which predicts potential cleavage of proteases for a given protein sequence, [72] TEM8 contained 59 predicted trypsin cleavage sites within the 564 amino acid protein sequence. A western blot was carried out to determine the optimal method of cell harvesting to maintain the integrity of cell surface TEM8. The data reveal non enzymatic cell dissociation reagent (Puck's-EDTA) was the optimal choice of reagent for maintaining protein integrity for functional assays. The blot in Figure 9 clearly shows that trypsin degrades TEM8 protein quite dramatically. All subsequent functional assays were carried out after harvesting of adherent cells with Puck's-EDTA to maintain protein integrity. Interestingly TEM8 appears as an 80-85 kDa "doublet" which is a little larger than expected. The difference is likely attributable to glycosylation, because treatment of cell extracts with a glycosidase cocktail reduced the size to 70kDa [54].

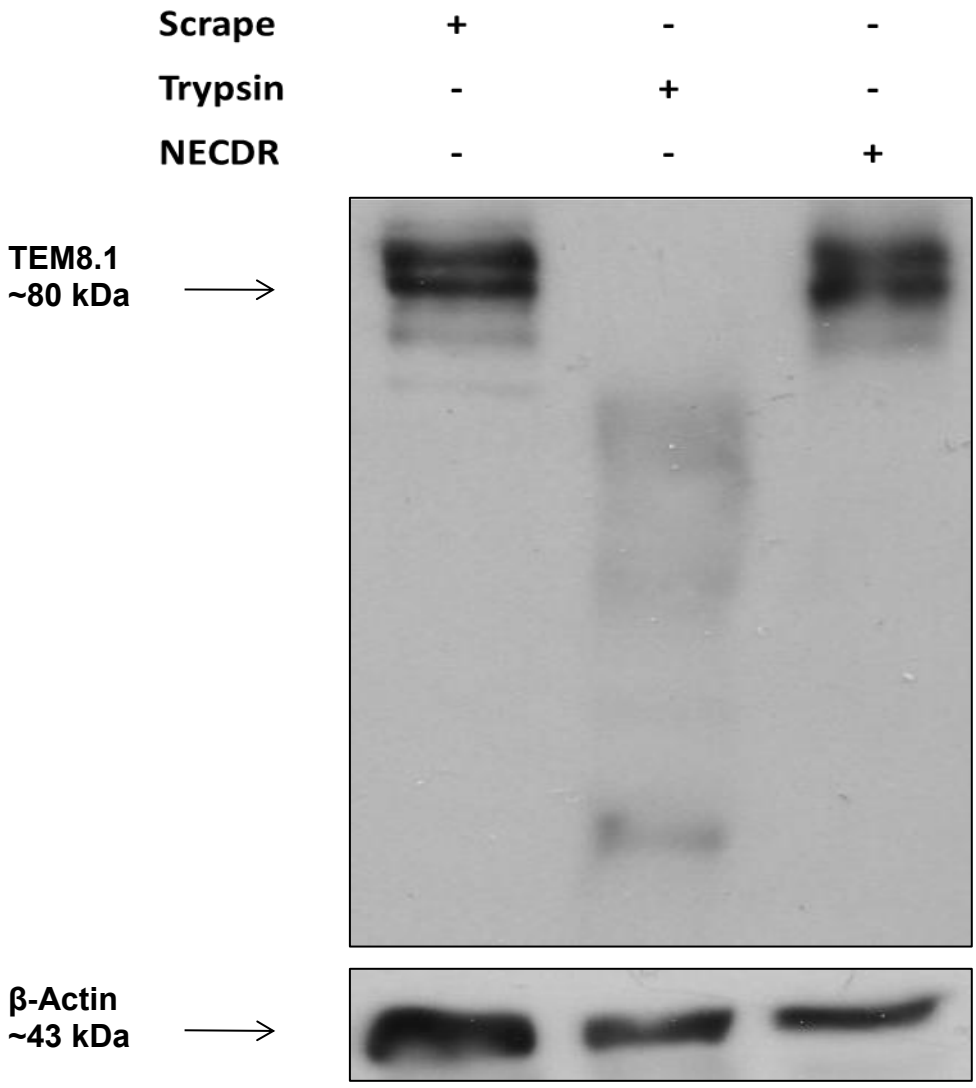

Figure 9 | Maintenance of TEM8.1 Protein Structure for Functional Assays

TEM8.1 was actively cleaved by trypsin, as it had 59 predicted trypsin cut sites (middle lane). Scraping of adherent cells,and using a Non Enzymatic Cell Dissociation Reagent (NECDR) Puck's/EDTA had no effect on TEM8 protein structure after cells were harvested.

5.1.2.3 Effect of TEM8 on Cellular Viability in the Presence of ECM Substrates

Tumor cell growth is dependent on both cell viability and cell death. Enhanced cellular viability is imperative to cancer cell survival [73], as this affords the opportunity for development of mutations that render certain cells resistant to therapy [74]. Moreover, with enhanced viability one could reasonably hypothesize that some of the resultant mutations may aid in the establishment of metastatic clones of cells over time thereby improving the likelihood of a metastatic event taking place. Cellular viability in MCF7, SKBR3, HTB20 pLentiTEM8 vs pLentiLacZ cell lines was carried out. Viability was assessed on BSA, Collagen-1, Fibronectin, and Matrigel. ECM substrates were used in the hope of stimulating TEM8 and causing downstream signalling events that may promote breast cancer cell viability.

Cellular viability was significantly enhanced in MCF7 cells over expressing TEM8 on Collagen-1 ($p<0.001$), Fibronectin ($p=0.0361$), and Matrigel ($p=0.0195$) when compared to control MCF7pLentiLacZ (Figure 10). In contrast, HTB20 and SKBR3 cell lines did not have enhanced viability on any ECM substrates (Figure 11, Figure 12). Perhaps this is due to lack of certain signalling elements within these cell lines that promote viability on ECM substrates through a TEM8 dependent mechanism. Another explanation could be related to the location of lentirvirus insertion into the genome; if for example the provirus interrupted a tumor suppressor or oncogene sequence.

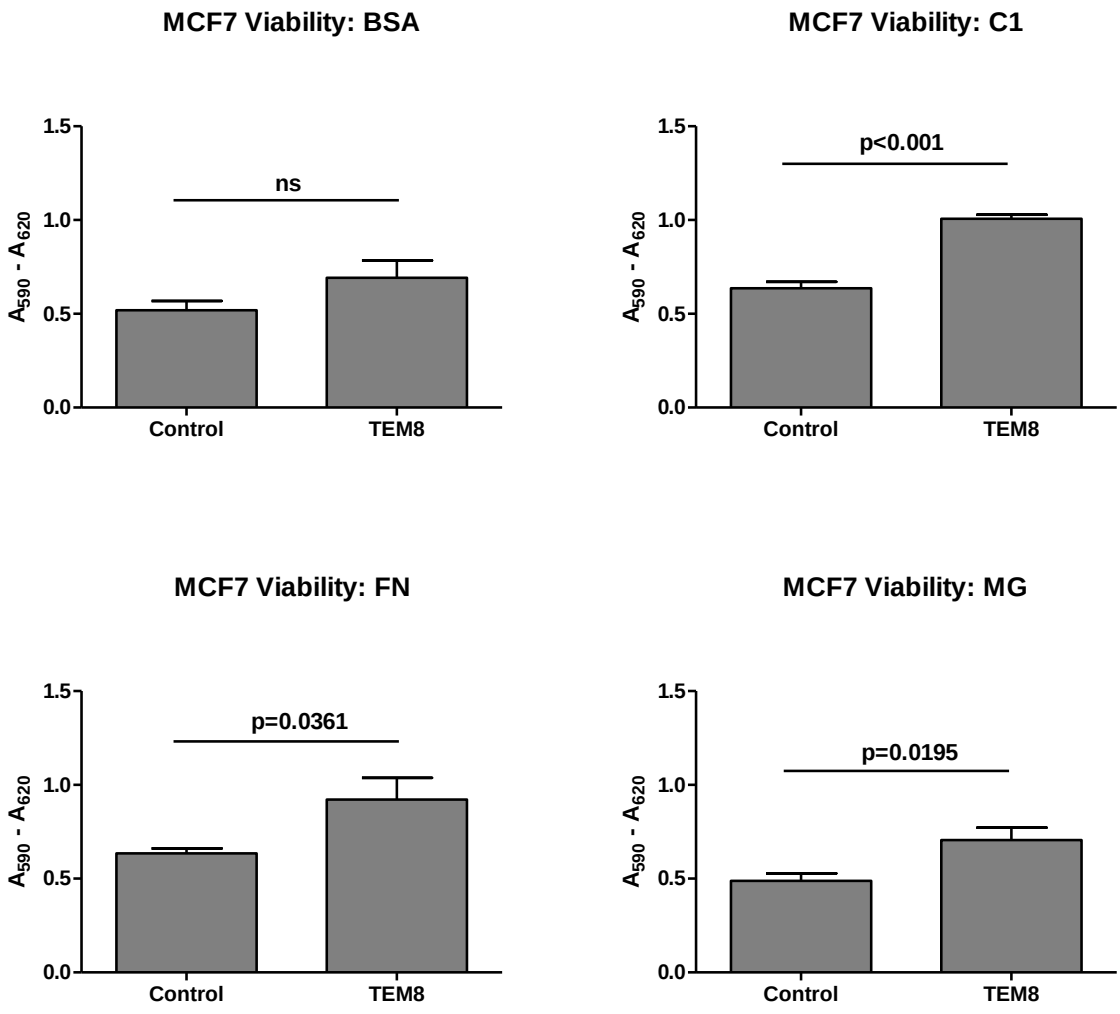

Figure 10 | A Comparison of MCF7pLentiLacZ and MCF7pLentiTEM8 cellular viability on Extracellular Matrix Substrates

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess viability in MCF7pLentiLacZ (control) and MCF7pLentiTEM8 cell lines after t=72h on various extracellular matrix substrates. MCF7pLentiTEM8 viability was enhanced on Collagen-1 (C1), Fibronectin (FN), and Matrigel (MG). Significance was computed using student t-test. Data are representative of two sets of experiments utilizing a non-enzymatic cell dissociation reagent.

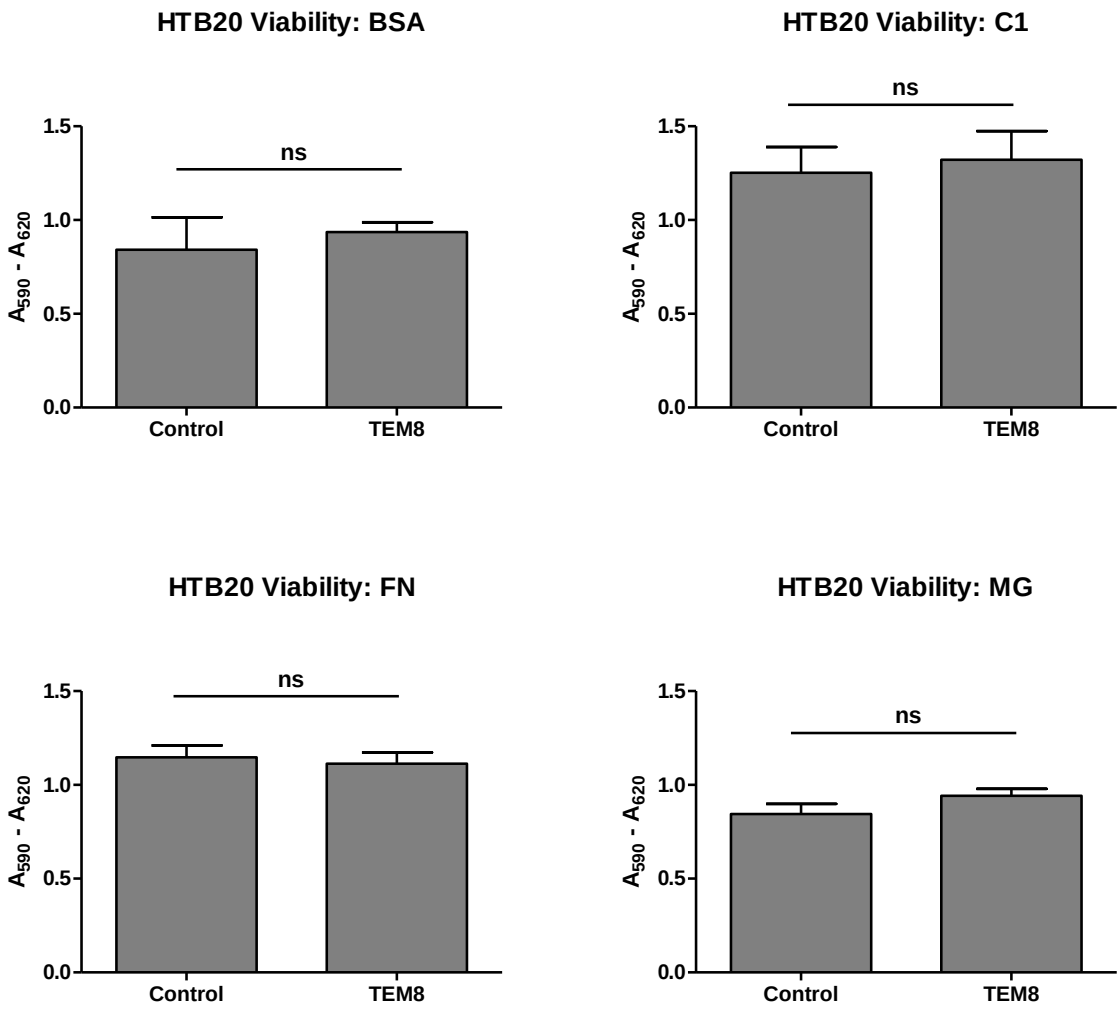

Figure 11 | A Comparison of HTB20pLentiLacZ and HTB20pLentiTEM8 cellular viability on Extracellular Matrix Substrates

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess viability in HTB20pLentiLacZ (control) and HTB20pLentiTEM8 cell lines after t=72h on various extracellular matrix substrates. HTB20pLentiTEM8 viability was not significantly enhanced on any of the ECM substrates tested. Significance was computed using student t-test. Data are representative of two sets of experiments utilizing a non-enzymatic cell dissociation reagent.

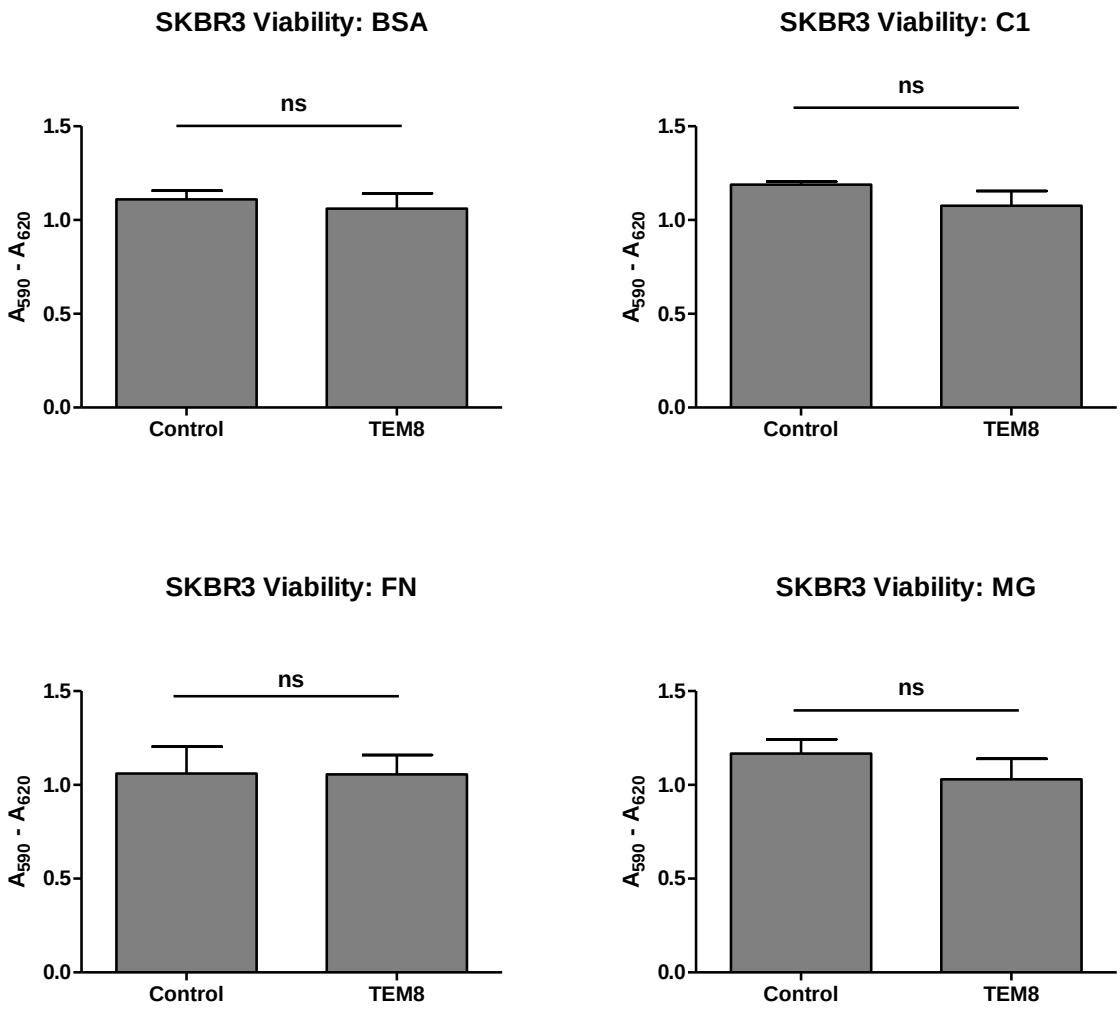

Figure 12 | A Comparison of SKBR3pLentiLacZ and SKBR3pLentiTEM8 cellular viability on Extracellular Matrix Substrates

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess viability in SKBR3pLentiLacZ (control) and SKBR3pLentiTEM8 cell lines after t=72h on various extracellular matrix substrates. SKBR3pLentiTEM8 viability was not significantly enhanced on any of the ECM substrates assayed. Significance was computed using student t-test. Data are representative of two sets of experiments utilizing a non-enzymatic cell dissociation reagent.

5.1.2.4 Apoptotic and Cell Cycle Consequences of TEM8

Tumor cell resistance to apoptosis is important in the development and progression of cancer. It was vital to determine whether TEM8 confers enhanced resistance to apoptosis. Although there was minimal change with cellular viability with overexpression of TEM8, it was important to look at apoptosis, as these processes are regulated by different factors. In MCF7, HTB20, and SKBR3 breast cancer cell lines there was no major difference in apoptosis between TEM8 and LacZ (Figure 13). It should be noted that there was a marginal increase in late apoptotic (FITC+/PI+) cells in SKBR3pLentiTEM8 vs. SKBR3pLentiLacZ with $p<0.05$. Also, there was a reduction in the proportion of necrotic (FITC-/PI-) cells in MCF7pLentiTEM8 compared to MCF7pLentiLacZ ($p<0.01$). No consistent differences in apoptosis amongst the breast cancer cell lines assayed were observed with overexpression of TEM8.

Alterations in cell cycle with respect to TEM8 were also considered. The connection between the cell cycle and cancer has been well established. The cell cycle is responsible for the control of cellular proliferation and apoptosis, with subsequent loss of regulation of the cell cycle leading to the development of cancer[75]. Cell cycle studies indicate, for all cell types, TEM8 does not change proportion of cells in G1, S, and G2/M (Figure 14).

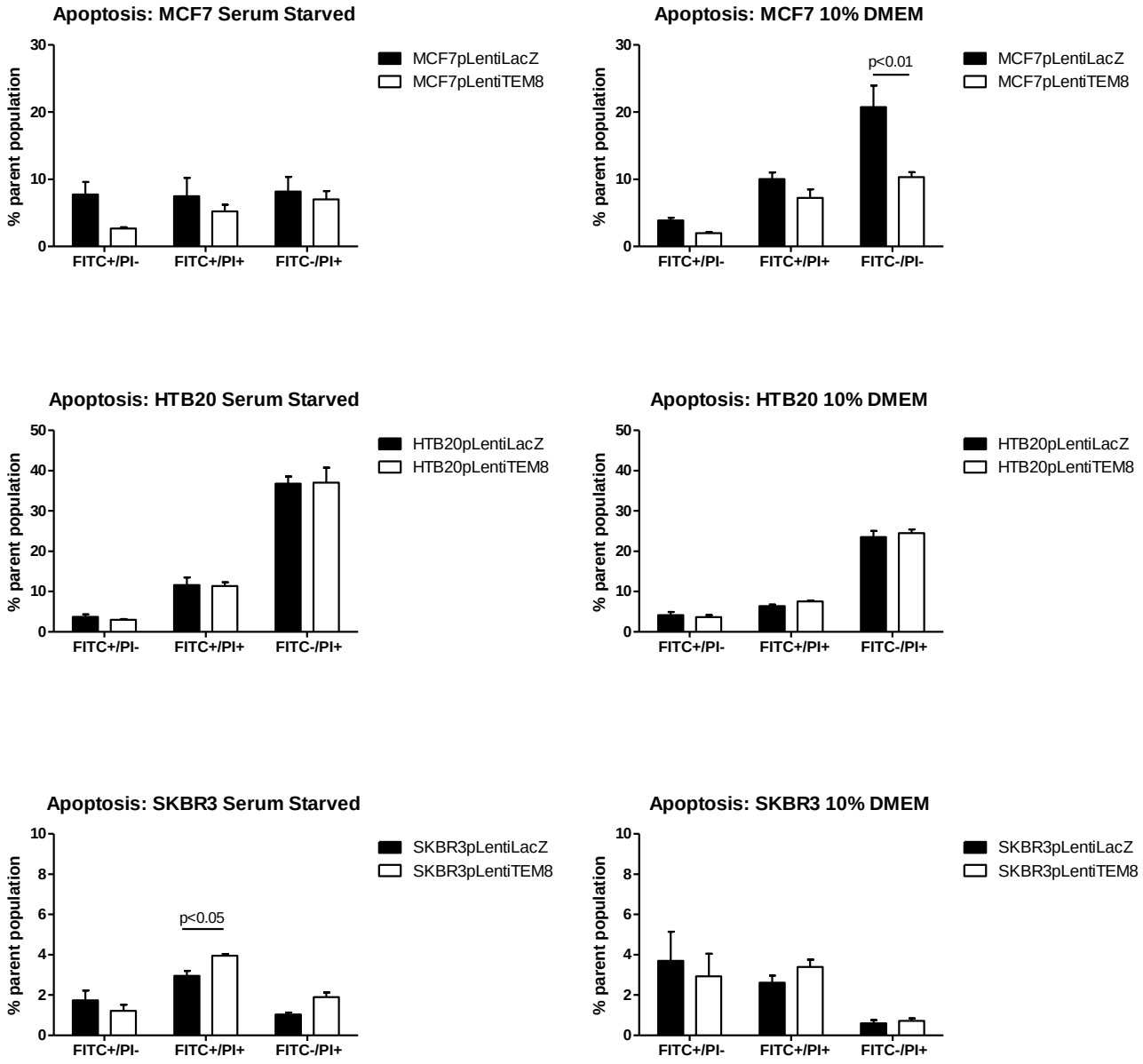

Figure 13 | Apoptosis in ± Serum Conditions in Human Breast Cancer Cell Lines Overxpressing pLentiLacZ vs pLentiTEM8

Apoptosis was assessed via Fluorescent Activated Cell Sorting (FACS). Cells were serum starved for t=48h or grown in 10% DMEM. Annexin FITC & PI were used to stain cells for FACS. Early Apoptosis (FITC+/PI-), Late Apoptosis (FITC+/PI+) and Necrosis (FITC-/PI+) were all evaluated. Two-way ANOVA was used to check for significance and interaction between all the variables tested; cell type and cell death. Bonferonni post-test was used to evaluate significance with α set to $p<0.05$.

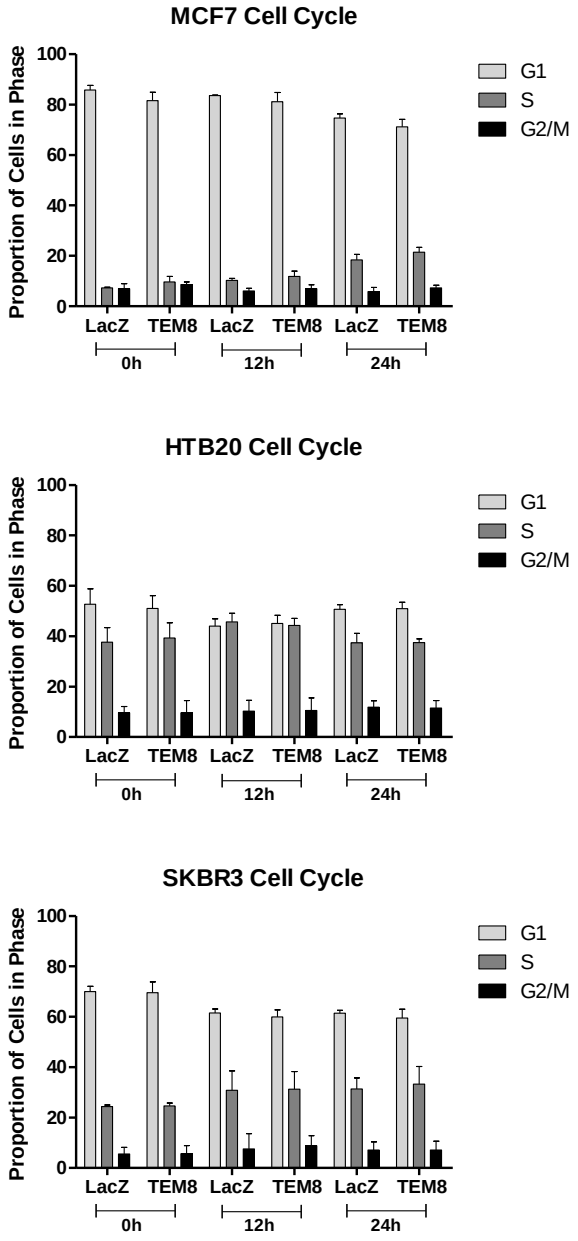

Figure 14 | Cell Cycle Analysis of Human Breast Cancer Cell Lines Overexpressing pLentiLacZ vs pLentiTEM8

Cells were synchronized via serum starvation and released and harvested at t=0h, t=12h, and t=24h. Cell cycle was analyzed by Propidium Iodide staining followed by Fluorescence Activated Cell Sorting (FACS).

5.1.2.5 TEM8 & Cellular Adhesion to the ECM

TEM8 contains a von Willebrand Factor A / Integrin Like Domain which suggests it may be involved in cellular adhesive processes [76]. Cancer cell - ECM adhesion is an important component of the metastatic process. ECM adhesion is typically modulated by integrin binding to extracellular matrix substrates, and this binding regulates downstream signaling cascades that are translated into dynamic cellular responses [77]. In an endothelial cell context, recent data indicate TEM8 is involved in adhesion to collagen-1 [53] and collagen-6 [54]. No data exist relating alterations in breast cancer cell adhesion to ECM in the context of TEM8.

Increases and decreases in cellular adhesion to the ECM were investigated with respect to TEM8 in MCF7, SKBR3, and HTB20 cells lines. To maintain structural integrity of TEM8, cells were harvested non enzymatically with Puck's/EDTA as previously described. Adhesion to BSA, Fibronectin, Collagen-1, Collagen-IV, Laminin-1, and Fibrinogen were all evaluated. The data indicate that with MCF7pLentiTEM8 there was enhanced adhesion to Fibronectin ($p<0.001$). TEM8 confers no change in adhesion in the HTB20 cell line on all ECM substrates tested. SKBR3pLentiTEM8 cell line had enhanced adhesion to Collagen-IV ($p<0.01$). There was no uniformity in terms of changes in adhesion amongst all cell lines with overexpression of TEM8 (Figure 15). This may be related to a lack of appropriate downstream signalling factors involved in TEM8 mediated adhesion to ECM substrates, or location of insertion of the lentiviral provirus into the host genome. MDA MB231 breast cancer cell line was used as a positive control for the adhesion assay.

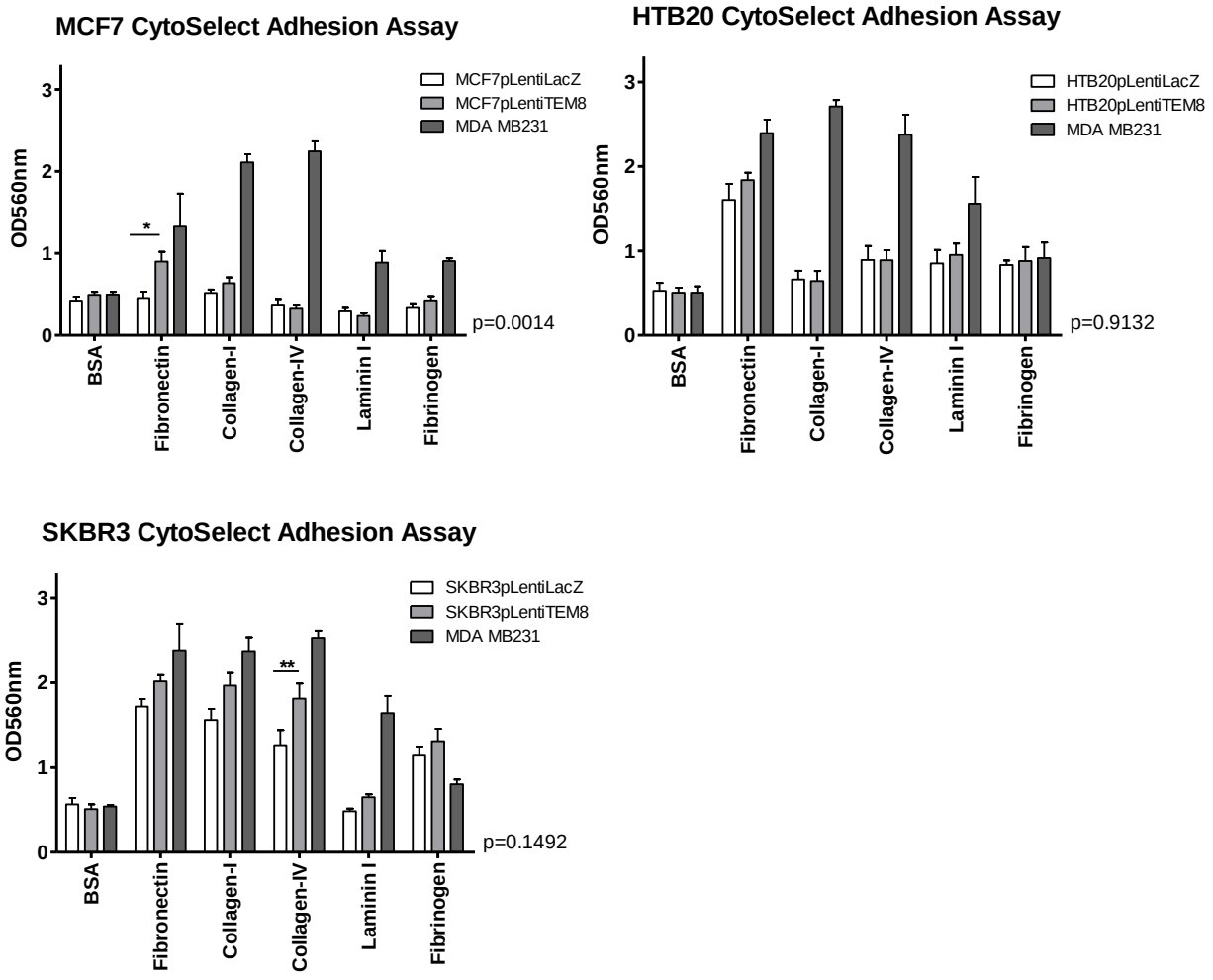

Figure 15 | Examining the Effect of TEM8 on Adhesion to various Extracellular Matrix Substrates

Using the Cytoselect adhesion assay the effect of TEM8 on adhesion to various ECM substrates was evaluated in MCF7, HTB20, and SKBR3 breast cancer cell lines with positive control MDA MB231. TEM8 significantly enhances adhesion to Fibronectin in MCF7 cell line ($p<0.01$). TEM8 did not significantly alter the adhesion of HTB20 cells to any of the ECM substrates assayed. SKBR3 cell lines had enhanced adhesion to Collagen-IV ($p=<0.001$). Two-way ANOVA was used to check for significance and interaction between all the variables tested; cell type and ECM substrate adhesion. Bonferroni post-test was then used to check for significance with α set at $p<0.05$. Note that $*p<0.05$, $**p<0.01$, $***p<0.001$. Data are representative of two experiments that utilized non-enzymatic cell dissociation reagent.

5.1.2.6 Breast Cancer Cell Migration is not impacted by TEM8 Over-Expression

Cellular migration is an important process of the metastatic cascade. Early stage cancer cells may exhibit enhanced motility along the inner ductal basement membrane during the initial stage of invasion into the underlying collagen-1 rich stroma [78]. For this reason, 2-D radial migration assays are effective in assessing cancer cell migration. TEM8-expressing endothelial cells migrate at a rate 3-fold greater than control cells in a monolayer denudation assay [42]. Examining the migratory effect of TEM8 in a breast cancer cell context could help determine whether or not TEM8 is involved in breast cancer migration. 2-D migration assays were carried out on tissue culture plastic, fibronectin, and collagen-1 in the hope that these ECM proteins may stimulate TEM8 signalling and lead to enhanced radial migration.

The results show that for all cell types there is no alteration in migration with overexpression of TEM8 on tissue culture plastic, fibronectin, and collagen-1 (Figure 16). Migration is regulated by a variety of factors including chemokines and other chemical factors [79]. The negative result may be due to lack of appropriate chemical signals thereby preventing TEM8 from eliciting its migratory effects within breast cancer cells. It may be that TEM8 does not enhance migration in the breast cancer cell lines tested.

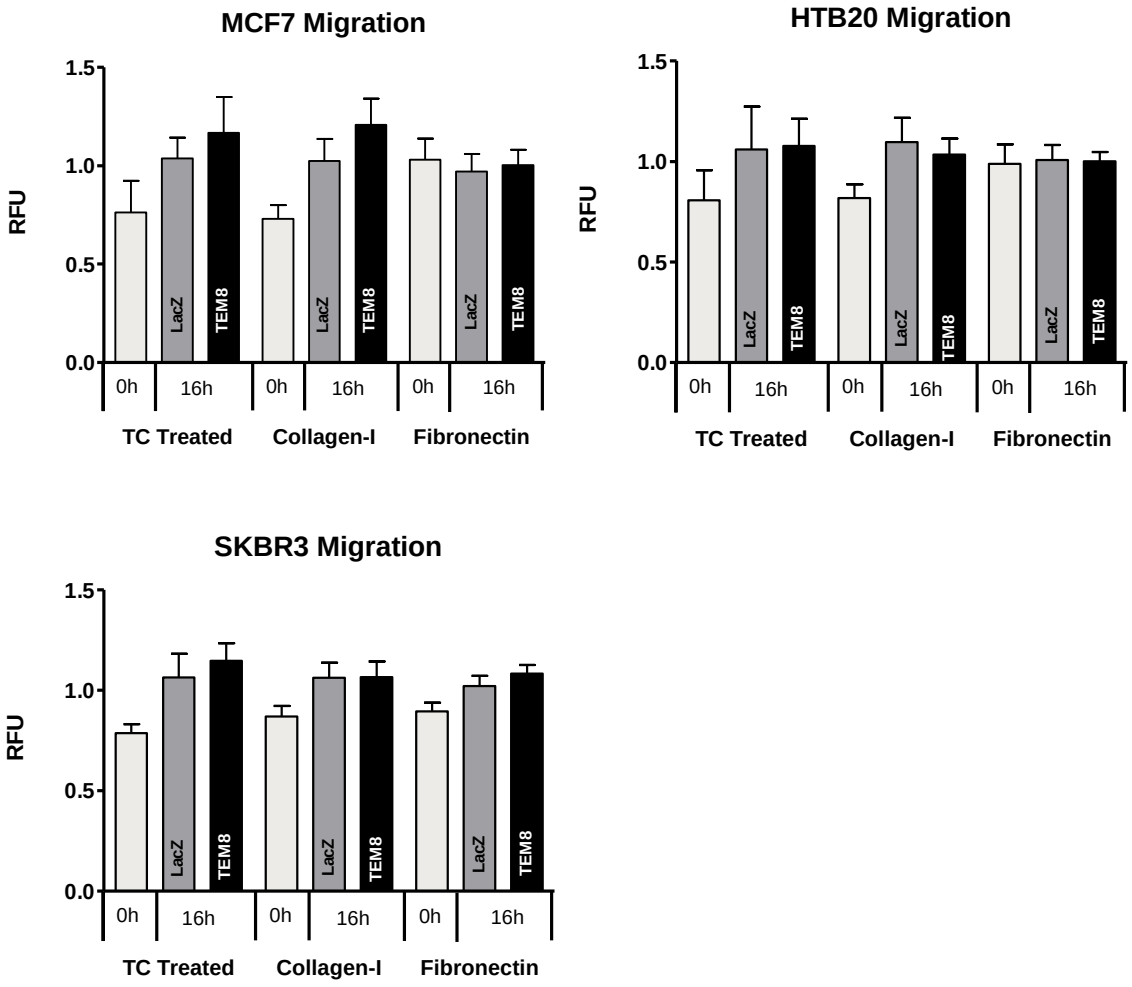

Figure 16 | Investigating the Consequence of TEM8 Expression on Breast Cancer Cell Migration

Utilizing Platypus Technologies Oris™ 2-D Cell Migration Assay the effect of TEM8 on cellular migration was investigated. Migration on Tissue Culture (TC) treated, Collagen-I, and Fibronectin coated wells was examined after t=16h. There was no significant change in migration on any of the substrates tested in MCF7, HTB20, and SKBR3 cell lines when control LacZ was compared to TEM8 infected cells. Significance was detected using Student t-test comparing LacZ to TEM8 at t=16h on each of the substrates assayed with α set to $p<0.05$. Data are representative of two experiments utilizing a NECDR.

5.1.2.7 Cellular Invasion is Not TEM8 Dependent

Breast cancer cell invasion is an important characteristic of cancer cells that confers enhanced metastatic propensity. There are a variety of factors influencing and facilitating breast cancer invasion. Breast cancer invasion arises from the accumulation of genetic alterations, the loss of cell-cell adhesion, and the process of epithelial-mesenchymal-transition. These processes facilitate the release of cancer cells from normal tissue architecture and are augmented by extracellular-matrix remodelling and other influences from the tumour microenvironment which culminate in cell migration. It is thought that coordination of many of these processes is likely to be required for breast cancer progression and invasion [80]. To infiltrate host tissues, changes in cancer cell adhesion and locomotion alone are not sufficient for penetration of ECM by the cancer cell. To breach both the basement membrane and ECM cells must produce proteolytic enzymes, such as MMPs, which allow for degradation of the ECM and movement through this barrier toward their final destination [17].

Utilizing 3-D culture assays the effect of TEM8 on breast cancer cell invasion was evaluated. The results indicate that TEM8 does not alter the invasive propensity of MCF7 (Figure 17), HTB20 (Figure 18), or SKBR3 (Figure 19) breast cancer cell lines. Invasion is a complex multi-step process as described above, and it appears that TEM8 alone does not alter the invasive phenotype of breast cancer cells.

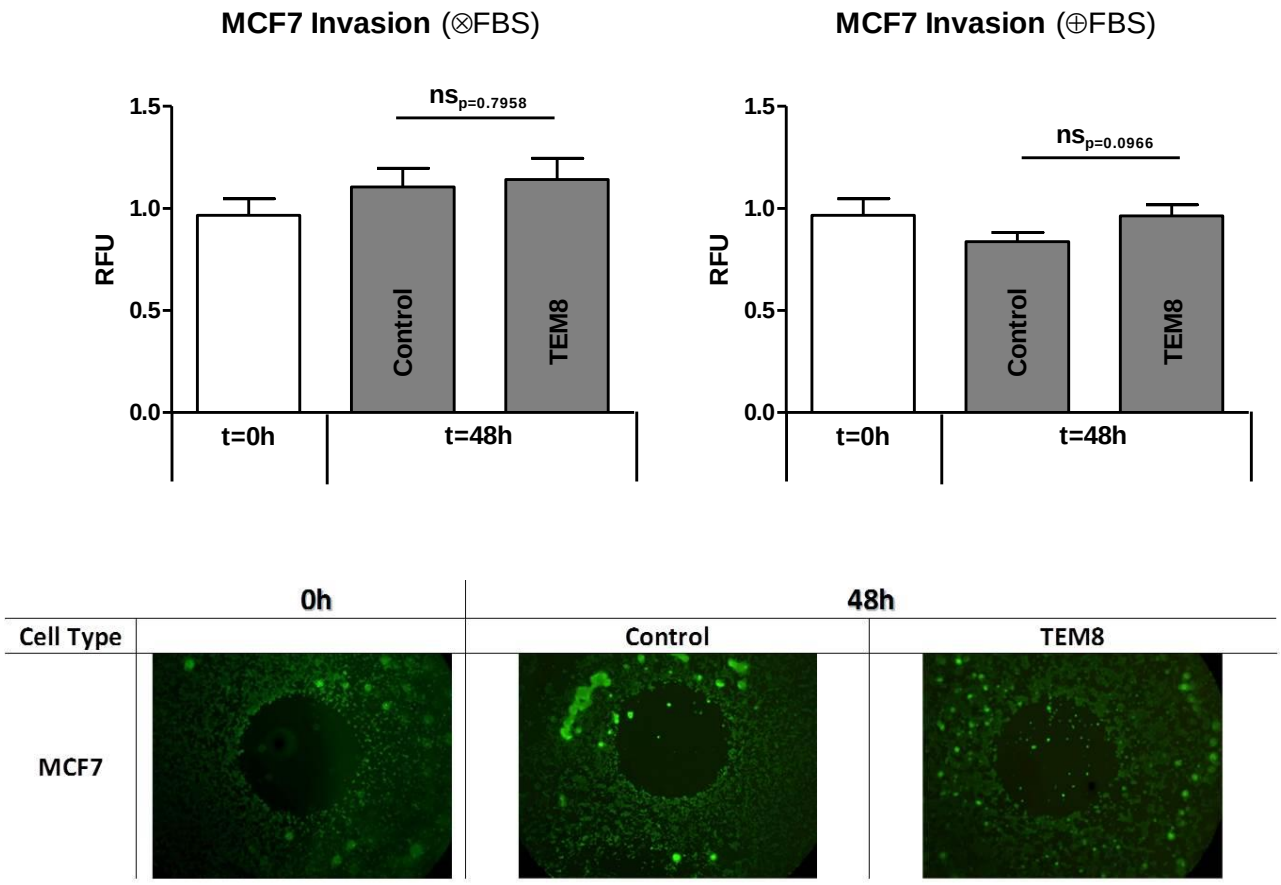

Figure 17 | Examining the Effect of TEM8 on Invasion in a Three Dimensional Culture Assay in MCF7 Breast Cancer Cells

The effect of TEM8 on cell invasion in adherent MCF7 cells was assayed. The assay was done in the presence or absence of Fetal Bovine Serum (FBS). The data suggest TEM8 does not confer enhanced invasive propensity in the MCF7 breast cancer cell line. Below the graph are representative images at t=0h and t=48h showing no difference in invasion between MCF7pLentiLacZ (control) vs MCF7pLentiTEM8. Significance was computed at t=48h using Student's t-test. Data are representative of two sets of experiments utilizing a non-enzymatic cell dissociation reagent.

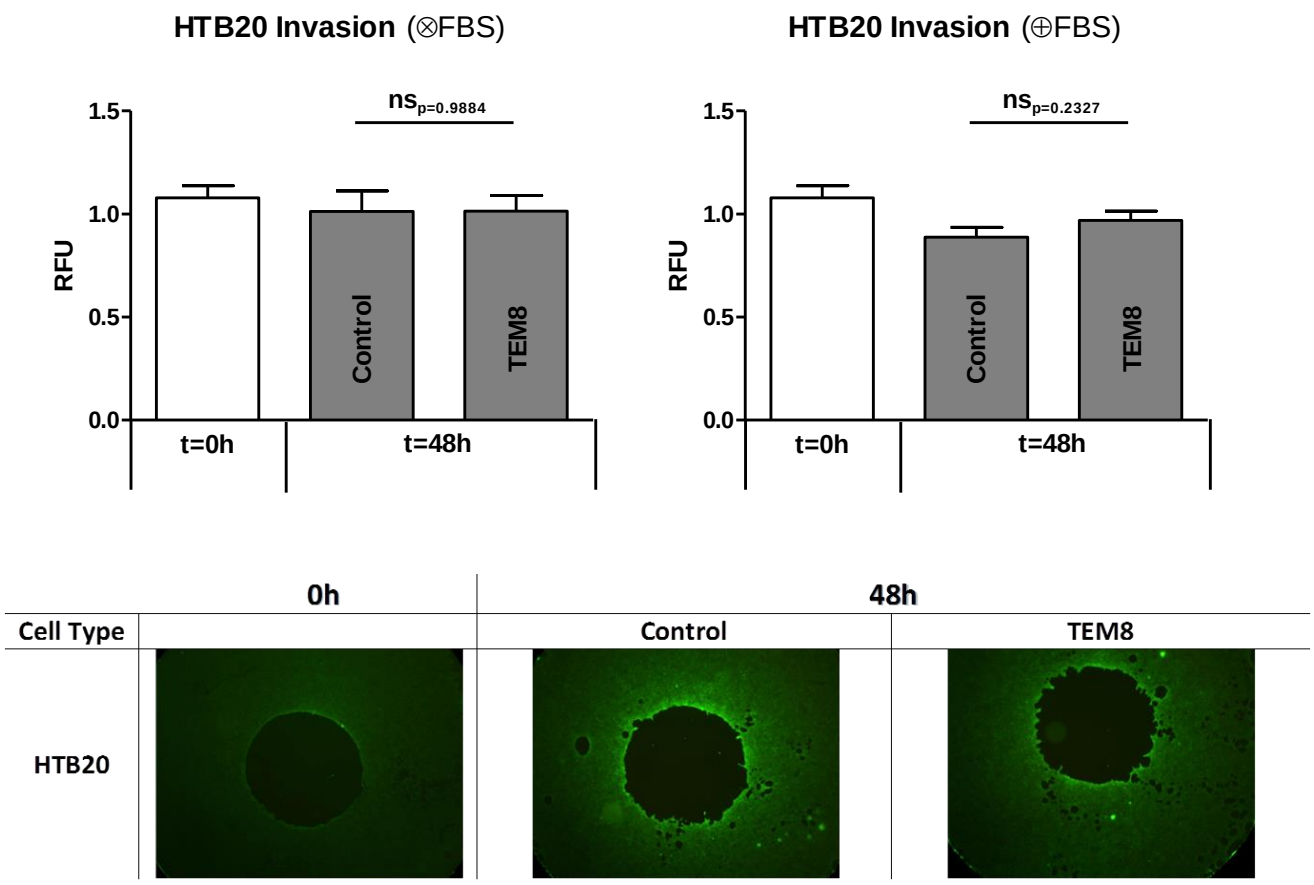

Figure 18 | Examining the Effect of TEM8 on Invasion in a Three Dimensional Culture Assay in HTB20 Breast Cancer Cells

Using Platypus Technologies Oris™ Cell Invasion 3-D Assay the effect of TEM8 on cell invasion in adherent HTB20 cells was assayed. The assay was done in the presence or absence of Fetal Bovine Serum (FBS). The data suggest TEM8 does not confer enhanced invasive propensity in the HTB20 breast cancer cell line. Below the graph are representative images at t=0h and t=48h showing no difference in invasion between HTB20pLentiLacZ (control) vs HTB20pLentiTEM8. Significance was computed at t=48h using Student's t-test. Data are representative of two sets of experiments utilizing a non-enzymatic cell dissociation reagent.

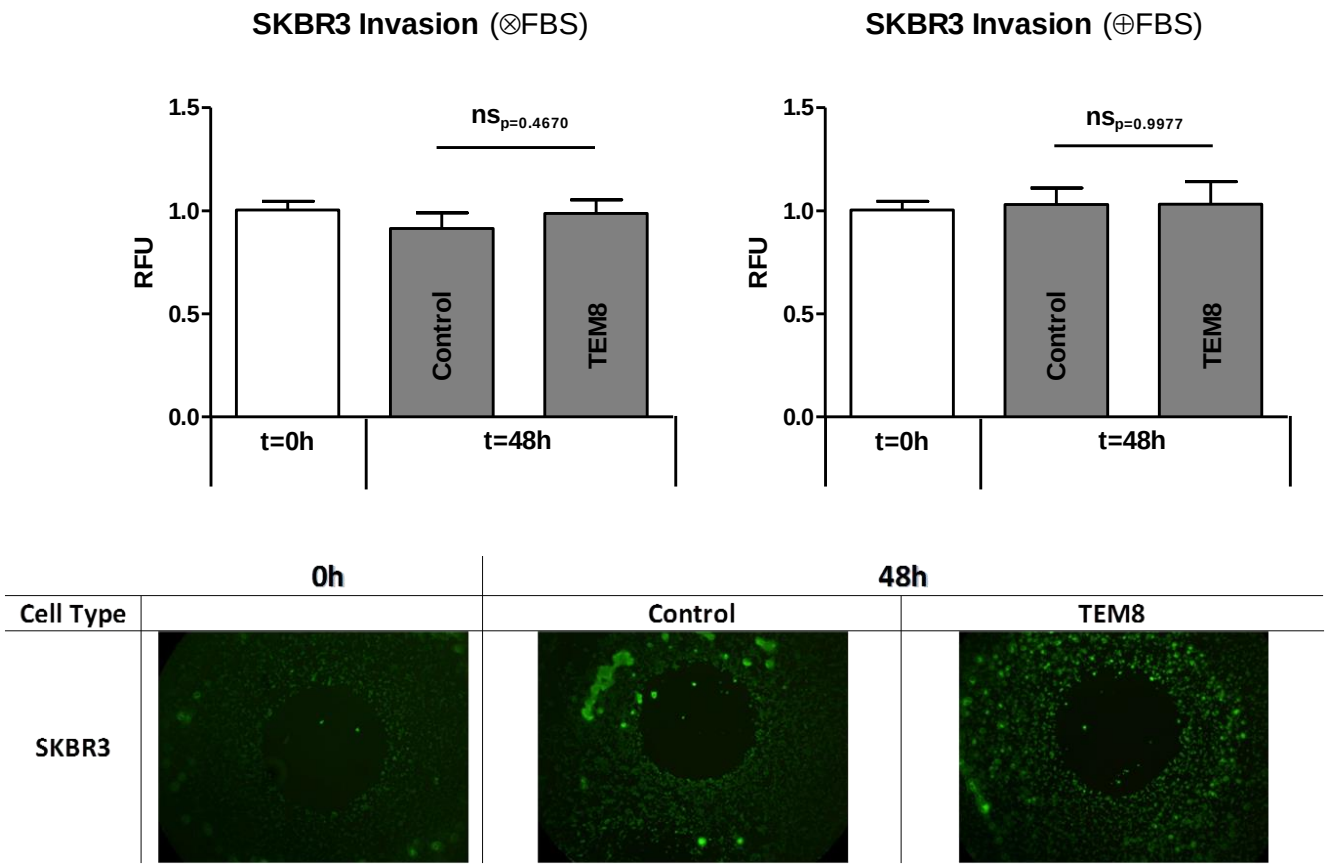

Figure 19 | Examining the Effect of TEM8 on Invasion in a Three Dimensional Culture Assay in SKBR3 Breast Cancer Cells

Using Platypus Technologies Oris™ Cell Invasion 3-D Assay the effect of TEM8 on cell invasion in adherent SKBR3 cells was assayed. The assay was done in the presence or absence of Fetal Bovine Serum (FBS). The data suggest TEM8 does not confer enhanced invasive propensity in the SKBR3 breast cancer cell line. Below the graph are representative images at t=0h and t=48h showing no difference in invasion between SKBR3pLentiLacZ (control) vs SKBR3pLentiTEM8. Significance was computed at t=48h using Student's t-test. Data are representative of two sets of experiments utilizing a non-enzymatic cell dissociation reagent.

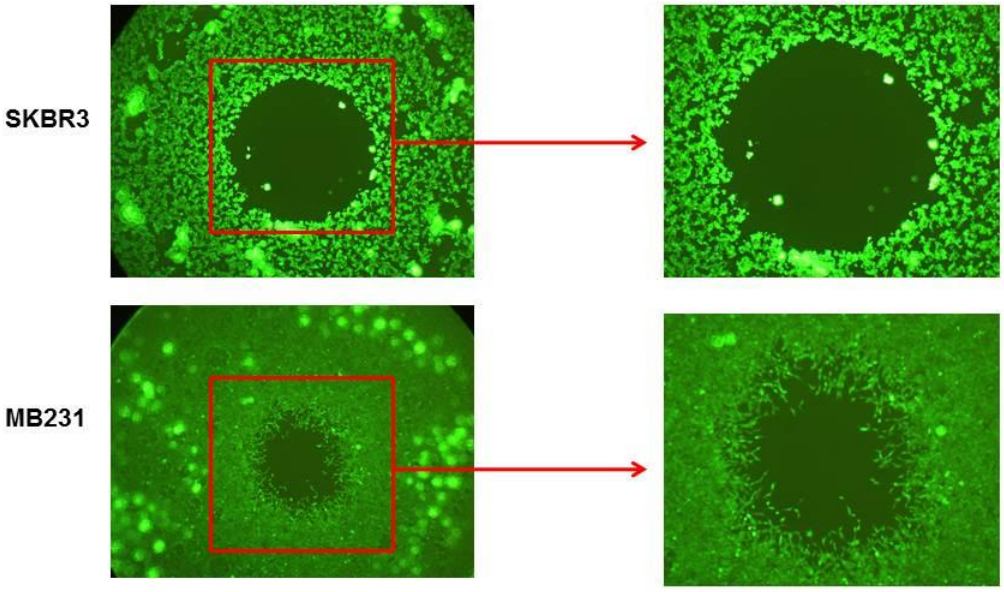

Figure 20 | Comparison of phenotypic change in SKBR3 and MDA-MB-231 breast cancer cells during 3D invasion assay

5.1.3 Chapter Discussion

The in vitro studies reveal TEM8 does not confer enhanced malignant behavior in MCF7, HTB20, and SKBR3 cell lines. TEM8 was effectively overexpressed in all three cell lines, as per western blot (Figure 8). Even though these cells were expressing TEM8, this does not indicate whether or not the protein is getting to the cell surface. To elicit TEM8 biological function it is imperative that it localizes to the cell surface so that it can interact with the ECM and other ligands.

Proteomic studies demonstrate direct interaction between the extracellular domain of TEM8 and M2 isoenzyme of protein kinase (M2PK) [81]. M2-PK, also known as tumor PK, participates in tumor cell growth and metastasis, and is released into peripheral circulation by tumor cells [82]. Surface TEM8 expression is imperative for appropriate interaction with ECM and signalling molecules like M2-PK. Use of immunofluorescence showed moderate TEM8 at the cell surface, but it should be noted that there was some background TEM8 detected in control LacZ cells. The SB5 TEM8 antibody donated by Brad St. Croix works well for western blot and immune precipitation without cross reactivity to other proteins [54]. SB5 is an IgG antibody that recognizes its epitope within amino acids 82 to 145. Unfortunately, the SB5 antibody fails to detect TEM8 at the cell surface in 293/TEM8, TEM8 positive endothelial cells, and CHO/TEM8 cells [83]. The discrepancy is likely due to the SB5 TEM8 epitope being masked because of ‘open and closed’ TEM8 conformations [83].

In retrospect, cell surface TEM8 could have been confirmed by purifying cell surface membrane proteins in pLentiTEM8 and pLentiLacZ to obtain crude membrane extracts free of cell debris and nuclei, followed by western blot to confirm the presence of TEM8 [84, 85]. Figure 8 shows that TEM8 was being expressed at a very high level in all cell lines. Perhaps, TEM8 was being overexpressed at too high a level making it biologically irrelevant; however, no system is perfect and cells are perturbed regardless of whether a pathway is disrupted by a knockout, a dominant gain-of-function mutation, or by overexpression [86]. Before experiments expression of TEM8 was confirmed via western blot, so lack of expression of TEM8 did not represent a potential source of error. The cells used in experiments were initially from single cell clones which reduces tumor cell heterogeneity, which may not accurately reflect in vivo intratumoral heterogeneity [87].

Experiments were initially optimized using trypsin as a cell dissociation reagent. The experiments with trypsin examining tumor cell viability, migration, and invasion show no significant differences between TEM8 overexpressors and controls. TEM8 is a type-1 transmembrane protein [76, 88] with 59 predicted trypsin cleavage sites. Western blot analysis confirmed that TEM8 was susceptible to trypsin digestion. TEM8 proteolytic cleavage may have interfered with TEM8 function. Assays were repeated using a non-enzymatic cell dissociation reagent (Puck's/EDTA). Even with the use of Puck's/EDTA cell scraping was required to get enough cells for experiments, so mechanical damage to cells cannot be entirely ignored.

Preliminary MTT assays on tissue culture treated dish did not yield any significant differences in viability. Breast cancer cell viability was assessed with a modified MTT assay on ECM substrates to stimulate cellular viability. For SKBR3 and HTB20 there were no differences in cellular viability on all substrata. MCF7 had enhanced growth on BSA, Collagen-I, Fibronectin, and non-specific Matrigel. Apoptosis evaluation via FACS revealed that when grown in 10%DMEM there was enhanced necrosis (PI+/FITC+) in MCF7pLentiLacZ when compared to MCF7pLentiTEM8. This apparent increase in the fraction of necrotic MCF7pLentiLacZ at t=48h, may explain the enhanced viability of MCF7pLentiTEM8 compared to LacZ on ECM substrates. No resistance to apoptosis with over-expression of TEM8 was noted in SKBR3 and HTB20 when grown in 10% DMEM. When TEM8 was knocked down in MDA-MB-231 with TEM8 shRNA vs control shRNA, cells grew at a similar rate in two dimensional culture and there was no difference in Survivin (regulator of apoptosis) expression [89].

TEM8 extracellular domain has been shown to mediate adhesion to collagen I [42] and collagen VI [54] in an endothelial cell context . There is no data relating TEM8 adhesion to the ECM in human breast cancer cells. Evidence relating TEM8 to cellular interactions with ECM proteins come from TEM8 knockout mouse studies. TEM8 KO mice are viable, however excessive deposits of extracellular matrix, particularly collagen, are found in many organs (ovaries, uterus, skin) in these mice. Moreover, female $TEM8^{-/-}$ mice have reproductive defects potentially related to deposits of ECM on the ovaries and uteri. The buildup of ECM in tissues of TEM8 KO mice reveal a potential role in ECM turnover. Our study shows minor differences in cellular adhesion with

overexpression of TEM8. MCF7 cells overexpressing TEM8 had increased fibronectin adhesion, and no reduction in adhesion to any of the ECM substrates. The premise of the design of the cytoselect adhesion assay was that reduced cellular adhesion to ECM likely aids metastatic spread [90]. However, enhanced adhesion may also promote metastasis since matrix-initiated signaling is sufficient to drive STAT3 activation, a reaction facilitated by EMT during breast cancer metastatic progression [91]. In SKBR3 over-expression of TEM8 enhanced adhesion to collagen IV. There were no changes in adhesion in HTB20s. There is no pattern with ECM adhesion as a function of overexpression of TEM8. It is difficult to draw conclusions with respect to adhesion as a function of TEM8 expression. Conservatively: in a breast cancer cell context TEM8 causes minor changes in adhesion depending on the breast cancer cell type assayed.

No differences in 2D radial migration or 3D invasion were noted. The migration and invasion assays on substrates are very artificial when compared to the in vivo situation. Our data show TEM8 does not promote migration on Collagen-I or Fibronectin, nor does it enhance invasion in a 3D artificial basement membrane gel. The invasion and migration experiments were optimized in the lab with MB-231 cell lines. Under the microscope MB231 cells adopted elongated cell morphology, and appeared polarized with loss of cell to cell adhesion (Figure 20). MDA-MB-231 breast cancer cells migrated and invaded well. By comparison, when MCF7, HTB20 or SKBR3 TEM8 vs LacZ cell lines were used, the cells appeared epithelial in nature, there was good cell to cell contact and virtually no observable or measureable movement. TEM8 did not provide the impetus for both migration and invasion on ECM substrates. Recent data from Chen et

al, has shown KO of TEM8 in TMD-231 cells reduces cellular invasion. Taken together TEM8 is likely necessary, but not sufficient for cellular invasion.

Compellingly, when TEM8 was overexpressed in normal mouse mammary epithelial cells morphological transformation suggestive of neoplastic change in 3D matrigel culture was observed [92, 93]. Conceivably, overexpression of TEM8 is an early event in breast cancer development, which helps enhance transformation of mammary epithelial cells to there more cancerous counterparts. Growth of mammary epithelial cells was enhanced with overexpression of TEM8 [93]. Additionally, TEM8 is expressed in normal stem cells and breast cancer stem like cells, with activation of TEM8 by a fragment of collagen VI alpha 3 increasing stem cell self-renewal in mammamosphere assays [89]. As it may be, TEM8 expression by breast cancer stem like cells may also be an early event in initiating tumorigenesis.

Although the in vitro data did not reveal any major functional change in tumor biology with overexpression of TEM8 we sought to explore whether a more complete tumor microenvironment, in vivo, was sufficient to elicit aggressive tumor cell biology with overexpression of TEM8.

CHAPTER SIX: TEM8 & IN VIVO TUMOR GROWTH

6.1 In Vivo Studies

6.1.1 Rationale

Previous work in our lab by Opoku-Darko et al. showed 4T1 cells infected with AdTEM8.1-GFP have increased tumor progression compared with control 4T1 AdGFP cells. Preliminary experiments were in a murine mammary carcinoma cell line, and the cells were not stably infected. An examination of tumor kinetics as a function of TEM8 expression in stably infected HTB20 human breast cancer cells was used to examine the effect of TEM8 on tumor growth *in vivo.* We also utilized a spontaneous MMTV-neu mouse tumor model comparing $TEM8^{+/+}$, $TEM8^{+/-}$, and $TEM8^{-/-}$ to more accurately reflect human biology.

6.1.2 Introduction

The in vitro data did not yield significant findings for TEM8 in the context of breast cancer cell function. We needed to better mimic the tumor microenvironment that the TEM8 infected breast cancer cells were interacting with, and did so by using a mouse model. A human-mouse xenograft model was used in which HTB20 cells were transplanted into non obese diabetic severely immunocompromised mice (NOD-SCID). Preliminary experiments had the highest success rate of effective tumor growth with the HTB20 cell line compared to MCF7 and SKBR3. The resultant tumors are a mosaic of human breast cancer cells and mouse stromal cells, and the environment more closely

resembles that seen in the human body when compared to the previously performed in vitro experiments.

6.1.2.1 Tumor Growth in ♀ NOD-SCID Mice

When HTB20pLentiTEM8 (n=13) vs HTB20pLentiLacZ (n=12) were injected into mammary fat pad of female NOD-SCID mice no change in tumor growth (Figure 21) was observed ($p = 0.9937$). The data unarguably indicate that TEM8 has no effect on tumor cell growth in vivo which is in agreement with the in vitro data previously described.

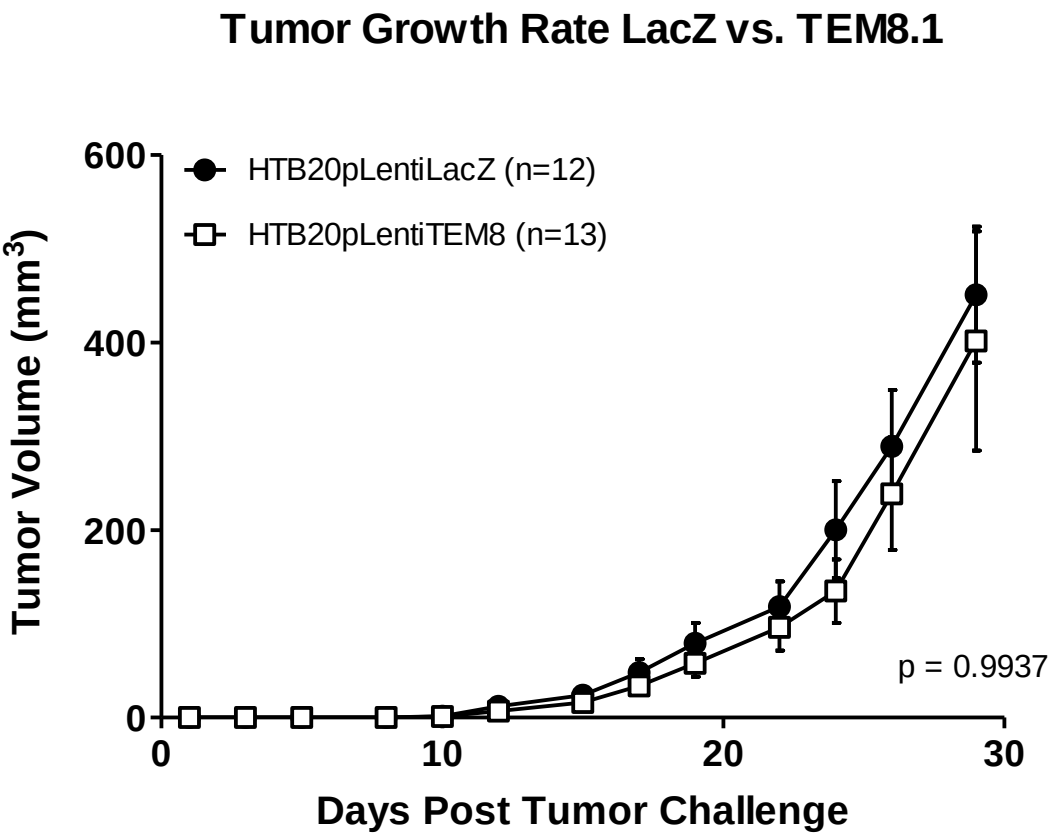

Figure 21 | Examining the Effect of TEM8 on Tumor Growth in Female NOD/SCID Mice

Female NOD SCID mice were challenged with either HTB20pLentiLacZ (n=12) or HTB20pLentiTEM8 (n=13) subcutaneously. Tumor growth rate was monitored thrice weekly. There was no significant difference in tumor growth rate in LacZ vs TEM8. Two-way repeated measures ANOVA was used to test for significance and interaction between variables cell type, tumor growth, and time.

6.1.2.2 Effect of TEM8 on Tumor Growth in MMTV-neu Mouse Model

Expression of the neu oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat results in the rapid induction of multifocal mammary tumors [94]. Activated neu transgene expression results in rapid conversion of the normal mammary epithelium to a malignant phenotype. MMTV-neu mice were used to evaluate the effect of TEM8 on breast tumor growth and mortality. $TEM8^{+/+}$, $TEM8^{+/-}$, and $TEM8^{-/-}$ strains of MMTV-neu mice were generated. Median survival for $TEM8^{+/+}$ was 173d, $TEM8^{+/-}$ 190d, and $TEM8^{-/-}$ 175.5d with p=0.8117. Our MMTV-neu mouse model revealed TEM8 does not alter median survival when $TEM8^{+/+}$ mice are compared to $TEM8^{-/-}$. Note that tumor size >1cm was the endpoint for this experiment, so it can be inferred that TEM8 does not affect tumor growth in vivo, which is in agreement with the NOD-SCID tumor challenge findings. These data demonstrate TEM8 expression in breast cancer cells is not enough of an impetus for enhanced tumor growth in vivo, however, there were only n=2 TEM8 KO mice (Figure 22).

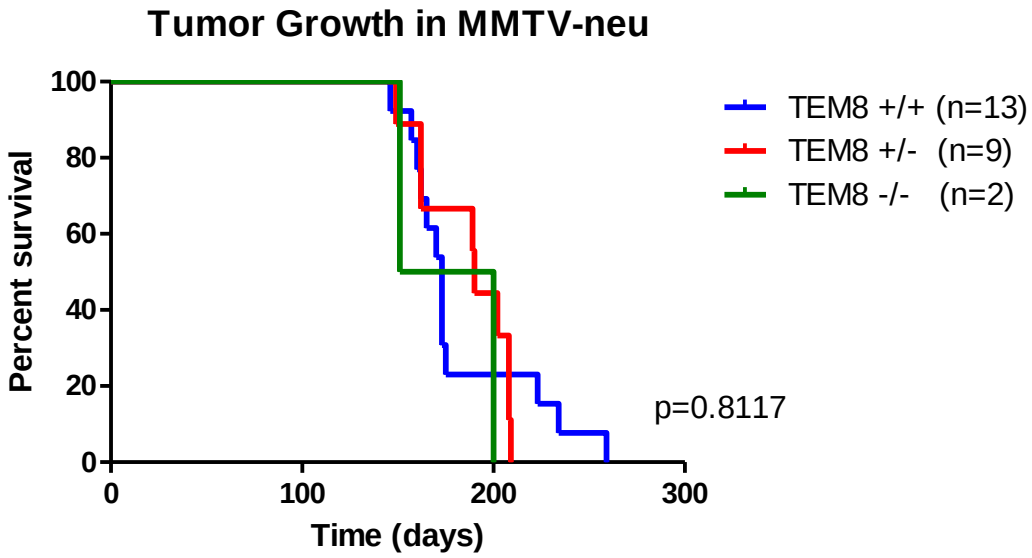

Figure 22 | Effect of TEM8 Knockout on Tumor Growth in MMTV-neu Mice

MMTV-neu mice carry an activated c-neu oncogene under the control of the mouse mammary tumor virus (MMTV) promoter which allows for the stepwise progression of carcinogenesis in mammary epithelium. MMTV-neu $TEM8^{-/-}$ knockout mice were constructed and tumor growth was compared to $TEM8^{+/-}$ and $TEM8^{+/+}$. Data show time to tumor > 1cm for $TEM8^{+/+}$ is 173d, $TEM8^{+/-}$ 190d, and $TEM8^{-/-}$ is 175.5d. Log Rank (Mantel Cox) test suggests no statistical difference in survival amongst the three groups of MMTV-neu mice.

6.1.3 TEM8 Expression in B6RIP-Tag

We were unable to generate sufficient MMTV-neu TEM8$^{-/-}$ mice due to breeding difficulties. Therefore, B6RIP-Tag mice were used to examine the effect of host derived TEM8 on tumor cell growth. The RIP-TAg transgene (rat insulin promoter, RIP, controlling the Simian Virus 40 large T antigen, TAg) confers a propensity to develop spontaneous insulinoma. Conveniently tumor burden can be monitored with blood glucose levels. Two back to back blood glucose readings of ≤4mmol/L (hypoglycemia) represent the experimental endpoint. Results were median survival of TEM8$^{+/+}$ 165d, TEM8$^{+/-}$ 197.5d, and TEM8$^{-/-}$ 191d. Log Rank (Mantel Cox) Test indicates the difference in survival between groups is significant with p=0.0086 (Figure 23).

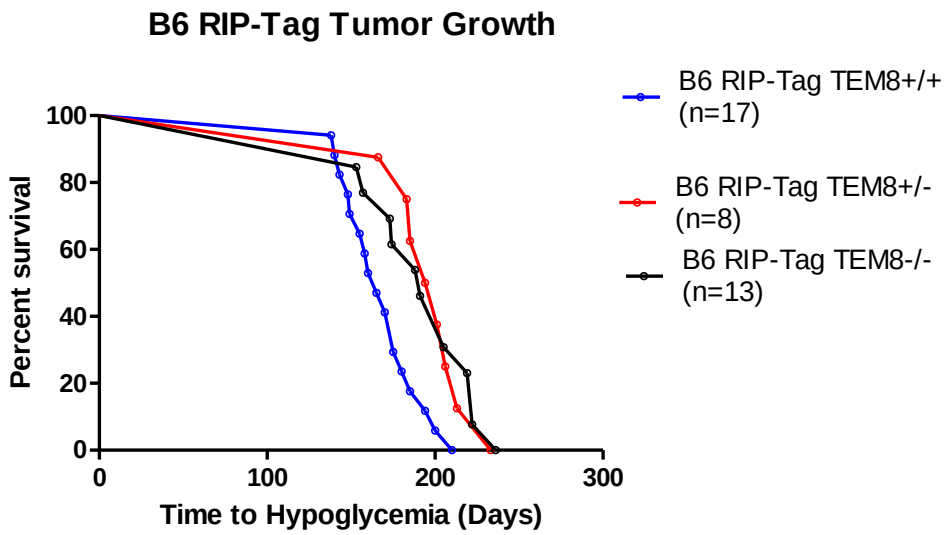

Figure 23 | Effect of TEM8 Knockout on Tumor Growth in B6-RIP-Tag Mice

B6 RIP-Tag develop spontaneous insulinomas, and tumor progression can be monitored via blood glucose levels. B6 RIP-Tag TEM8 -/- mice were constructed and survival was compared to $TEM8^{+/+}$ and $TEM8^{+/-}$. Mice were sacrificed, or tumor burden was deemed too large, when blood glucose levels were represented by two readings ≤4mM. Median survival of $TEM8^{+/+}$ was 165d, $TEM8^{+/-}$ 197.5d, and $TEM8^{-/-}$ 191d. Log Rank (Mantel Cox) Test indicates that the difference in survival is significant with p=0.0086.

6.1.4 Chapter Discussion

Overexpression of TEM8 in HTB20 breast cancer cells did not confer enhanced tumor growth in female NOD-SCID mice. The result is not surprising since in vitro results showed no enhanced viability or resistance to apoptosis in this cell line. TEM8 knockout mice challenged with MDA-MB231 orthotopically in the mammary fat-pad show inhibited tumor growth compared to wild type littermate controls [95]. Tumor growth was inhibited in TEM8 KO mice when challenged with melanoma, breast, lung, and colon cancer indicating that host derived TEM8 promotes the growth of certain tumors [49, 95]. When our overexpression mouse studies are taken together with the TEM8 KO in vivo studies, it further suggests that host TEM8 expression is necessary for tumor growth, but not sufficient.

Review of the literature shows TEM8 expression primarily in the endothelial cells of tumor vasculature in colorectal and breast cancer [32, 54, 96], and not the tumor cells themselves. Histological studies of tumors treated with TEM8 targeting antibodies and fusion proteins reveal reduced vascularity [69, 81, 95, 96]. CD31 vessel staining of DLD1 (colon carcinoma cell line) tumors in mice xenografts have reduced vessel density in TEM8 KO mice. Reduced microvessel density is also observed in TEM8 antibody treated mice challenged with DLD-1 human colon carcinoma cells revealing a role for TEM8 in tumor angiogenesis. FITC-labelled TEM8 antibody injected IV into DLD1 tumor bearing mice revealed localization of TEM8 selectively in tumor associated vasculature [95]. Moreover, MCF7 and LS-180 tumors treated with a TEM8-Fc fusion protein have inhibited growth and reduced microvessel density [81]. Finally, a DNA

vaccine against TEM8 protected mice from lethal challenges against tumor cells, reducing tumor growth and increasing lifespan, with significant suppression of angiogenesis in tumors observed[69]. All of these data point out that disruption of TEM8 expression directly interferes with the tumor vasculature, and this changes tumor biology. It is likely not expression of TEM8 by the tumor cells themselves causing enhanced growth and metastasis, but rather TEM8 expression by the tumor vasculature.

Furthermore, liver metastases were reduced in MCF7 athymic nude mice xenografts when treated with TEM8-Fc fusion protein [81]. One can speculate that this reduction in metastasis is due to the reduced vascularity of these TEM8-Fc treated tumors which would theoretically diminish the chance of haematogenous tumor cell dissemination to a distant site. Lastly, histological studies of TEM8 in triple negative breast cancer (TNBC) display high level of TEM8 in adjacent stroma to TNBC and in between tumor cells. None of the TNBC cases showing immunoreactivity for TEM8 was in the epithelial tumor cells [48].

Recently TMD-231 breast cancer cells, which spontaneously metastasize to lung, were treated with TEM8 shRNA producing smaller primary tumors in nude mice. Interestingly there was no difference histologically when compared to control shRNA. Reduced growth was not observed in vitro with TEM8shRNA in TMD-231 cells [89]. The differences in growth rate in vivo could be related to the ability to establish and/or respond to the proper tumor microenvironment.

In the same experiment there was reduced lung metastasis in TMD-231 shTEM8 knock down. In vitro the reduced distant metastasis was attributed to a lower invasive capacity demonstrated by matrigel invasion assay [89]. These data divulge TEM8 as necessary for invasion in vitro and in vivo, but our experiments suggest it is *not sufficient* when overexpressed independently.

We attempted to make MMTV-neu TEM8 knockout mice. MMTV-neu TEM8 KO mice were constructed, but due to breeding difficulties, only n=2 MMTV-neu TEM8 KO mice were generated. Breeding difficulties of $TEM8^{-/-}$ mice are reported in the literature and explained by changes in ECM homeostasis which causes excessive extracellular matrix buildup on the ovaries of females [49, 97]. There was no difference in mortality, defined as tumor >1cm in any dimension, between $TEM8^{+/+}$, $TEM8^{+/-}$, and $TEM8^{-/-}$. This is likely due to the low numbers of mice in the study. Deriving any definitive conclusion from these data is difficult.

B6 RIP-Tag transgenic mice which develop spontaneous insulinoma had reduced survival when host derived TEM8 was present. This was expected since TEM8 likely promotes tumor angiogenesis which enhances tumor growth and metastasis. B6 RIP-Tag TEM8 KO mice had enhanced survival which was also expected, since knocking out TEM8 likely interferes with tumor angiogenesis and subsequent tumor growth and metastasis. The $TEM8^{+/-}$ B6 RIP-Tag had improved survival similar to that seen in the $TEM8^{-/-}$ mice which was unexpected. $TEM8^{+/-}$ mice still have one TEM8 allele which would allow for

expression of TEM8 in these mice. The level of expression of TEM8 in B6 RIP-Tag TEM8$^{+/-}$ mice was never quantified.

CHAPTER SEVEN: DISCUSSION AND FUTURE DIRECTIONS

7.1 Thesis Overview

This thesis aimed to study the role of tumor endothelial marker 8 in human breast cancer cells, and characterize its function. Previous work from our lab demonstrated transient overexpression of TEM8 in murine 4T1 mouse mammary carcinoma cells enhanced tumor growth and metastasis to the lymph nodes and lung. TEM8 expression in breast cancer was found to be dichotomous with basal subtype cancer cells expressing high levels, and luminal subtype low levels. Based on these findings, this project was designed to study TEM8 in stably infected, human breast cancer cells of the luminal subtype. The study objectives stated in the intro of this thesis were:

1. The prognostic significance of overexpression of TEM8 in human breast cancer cells will be delineated.
2. The in vitro characteristics of TEM8 overexpression in breast cancer cells will be evaluated
3. The in vivo characteristics of TEM8 overexpression in breast cancer cells will be evaluated

Initially the prognostic significance of TEM8 was evaluated in a homogenous population of tumor cells using laser capture micro dissection (LCM). Lymph node positive and lymph node negative clinical samples were compared. Technically this proved very difficult, and was unsuccessful. Instead, MediSapeins provided an in silico molecular pathology report for TEM8 which was extensively analyzed. The findings suggest that

increased TEM8 expression is associated with lymph node positivity in breast cancer. Gutwein et al. have noted that there is increased TEM8 expression in axillary LN specimens from patient with loco regional metastasis [48]. Unfortunately they were not able to identify which cells were overexpressing TEM8, and they hypothesize TEM8 expressing cells were stromal cells that had travelled with tumor cells to the axillary lymph nodes [48]. Overexpression of TEM8 does not predict distant metastasis, but it is associated with increased incidence of metastasis to bone (Figure 6). No difference in survival between high and low TEM8 expressers were noted.

The in vitro studies were carried out using a non-enzymatic cell dissociation reagent to prevent TEM8 digestion; we discovered TEM8 had multiple trypsin digestion sites. The in vitro studies did not reveal any major function for TEM8 in luminal subtype breast cancer cells. Enhanced growth in MCF7 cells on ECM substrates with overexpression of TEM8 was likely due to an increase in the population of necrotic MCF7pLentiLacZ cells as seen in the apoptosis studies. Moreover, there was no consistent finding with respect to adhesion to extracellular matrix substrates which was unexpected considering TEM8 is an adhesion molecule with known ligands[42, 53, 54, 98]. No differences were noted in migration or invasion either. Migration and invasion were evaluated using well characterized commercially available assays [99, 100] in both two and three dimensions.

In vivo tumor growth studies were used in the hope that the introduction of a more sophisticated microenvironment may augment TEM8 activation/signalling. HTB20pLentiTEM8 and HTB20pLentiLacZ tumors grew at similar rates, and no

difference was noted. Tumor growth was not explored in the other cell lines based on this finding and the in vitro data. MMTV-neu TEM8 KO mice were constructed, but breeding difficulties were encountered. There was no difference in mortality among $TEM8^{+/+}$, $TEM8^{+/-}$, and $TEM8^{-/-}$. This is likely due to the low numbers of mice in the study. B6 RIP-Tag $TEM8^{-/-}$ and $TEM8^{+/-}$ mice had improved survival compared with wildtype B6 RIP-Tag $TEM8^{+/+}$ mice. TEM8 expression in $TEM8^{+/-}$ mice was never quantified which complicates interpretation of the data.

When we look at the data as a whole there is very little evidence relating overexpression of TEM8 with changes in the biological behavior of non-invasive breast cancer cells of the luminal subtype. The reasons for this could be multifactorial. The in vitro assays do not provide a sufficiently complex microenvironment so lack of an appropriate TEM8 ligand may be an issue. Also, cell surface expression of TEM8 was difficult to demonstrate with lack of an appropriate IF and IHC antibody, and stochastically, TEM8 may have been expressed at too high a level. Interestingly we discovered an association between expression of TEM8 and lymph node positivity in breast cancer tissue samples. The association between TEM8 and lymph node positivity also held true for colorectal cancer, lung adenocarcinoma, and gastric adenocarcinoma (Figure 24-26).

7.2 TEM8 function in Breast Cancer

Tumor vasculature has enhanced TEM8 expression on endothelial cells of multiple tumor types [32, 54, 96]. Targeting TEM8 in pre-clinical studies has revealed reduction in tumor angiogenesis, growth, and metastasis. Tumors treated with the TEM8-Fc antibody

[81], TEM8 antibodies [95], and TEM8 DNA vaccines [69] have reduced blood vessel density which likely explain anti-tumor effects and reduced metastasis. Tumor xenografts are impaired in TEM8 KO mice indicating TEM8 promotes the growth of human tumor xenografts [95]. Most all information available in the literature point to a role for TEM8 in tumor endothelial cells. Overexpression of TEM8 in luminal subtype breast cancer cells is not sufficient for the development of a more malignant phenotype. Nonetheless, TEM8 is likely necessary, since knockdown in TMD231 cells reduces invasiveness [89].

TEM8 is a cell surface receptor that plays an important role in promoting tumor angiogenesis. As such it is likely a good target for novel anti angiogenic therapeutics. Overexpression of TEM8 in luminal breast cancer cells is not sufficient for altering the biological behavior in vitro and in vivo. Although studies of both mouse and human tissues document some expression of TEM8 within tumor cells, the most abundant expression of TEM8 is seen within tumor vasculature and stroma.

7.3 Future Directions

An association between expression of TEM8 and lymph node metastasis was discovered. Previously we attempted to use LCM to isolate tumor cells from LN positive and LN negative tissue samples on a microscope slide. Obtaining good quality RNA from LCM samples was difficult. Alternatively, these tissue samples could be disrupted, homogenized, and then western blotted for TEM8. The difficulty in this is that we will have a heterogeneous population of endothelial, stromal, and tumor cells such that

increases in TEM8 cannot be attributed to tumor cells alone. More simply, IHC/IF for TEM8 in these tissue samples would be useful in determining the localization of TEM8. Double staining for TEM8 with CD31 and/or cytokeratin would localize TEM8 to the vascular endothelium or tumor cells of epithelial origin [101]. Unfortunately, the SB5 TEM8 antibody provided to us is ineffective for IF and IHC; however, in 2012 a new TEM8 antibody, AF334, was developed for IF [83].

The in vitro studies reveal TEM8 overexpression was not sufficient to make MCF7, HTB20, or SKBR3 more malignant in their biological behavior. With that being said, our initial results showed dichotomous TEM8 expression. Here we have studied the low TEM8 expressor luminal subtype breast cancer cells with overexpression of TEM8. In reverse, high TEM8 expressing (MDA MB-231, MB-468, MB-436, Hs578T) breast cancer cells of the basal lineage could have knockdown of TEM8. Knocking down TEM8 using commercially available siRNAs followed by functional studies may reveal TEM8 dependent functions.

7.4 Closing Remarks / Conclusions

The work in this thesis has demonstrated that TEM8 minimally contributes to altering biological behavior of breast cancer cells of luminal subtype. The literature suggests that TEM8 is specific to the tumor vasculature and plays a major role in tumor angiogenesis. Overexpression of TEM8 in mouse mammary epithelial cells causes malignant transformation in vitro [92]. Imaginably, TEM8 is an early regulator of neoplastic change in mammary epithelial cells, as well as a regulator of tumor specific angiogenesis

in tumor associated endothelial cells. Most importantly we have discovered that in breast cancer there is an association between TEM8 expression and lymph node positivity.

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CHAPTER EIGHT: APPENDIX I

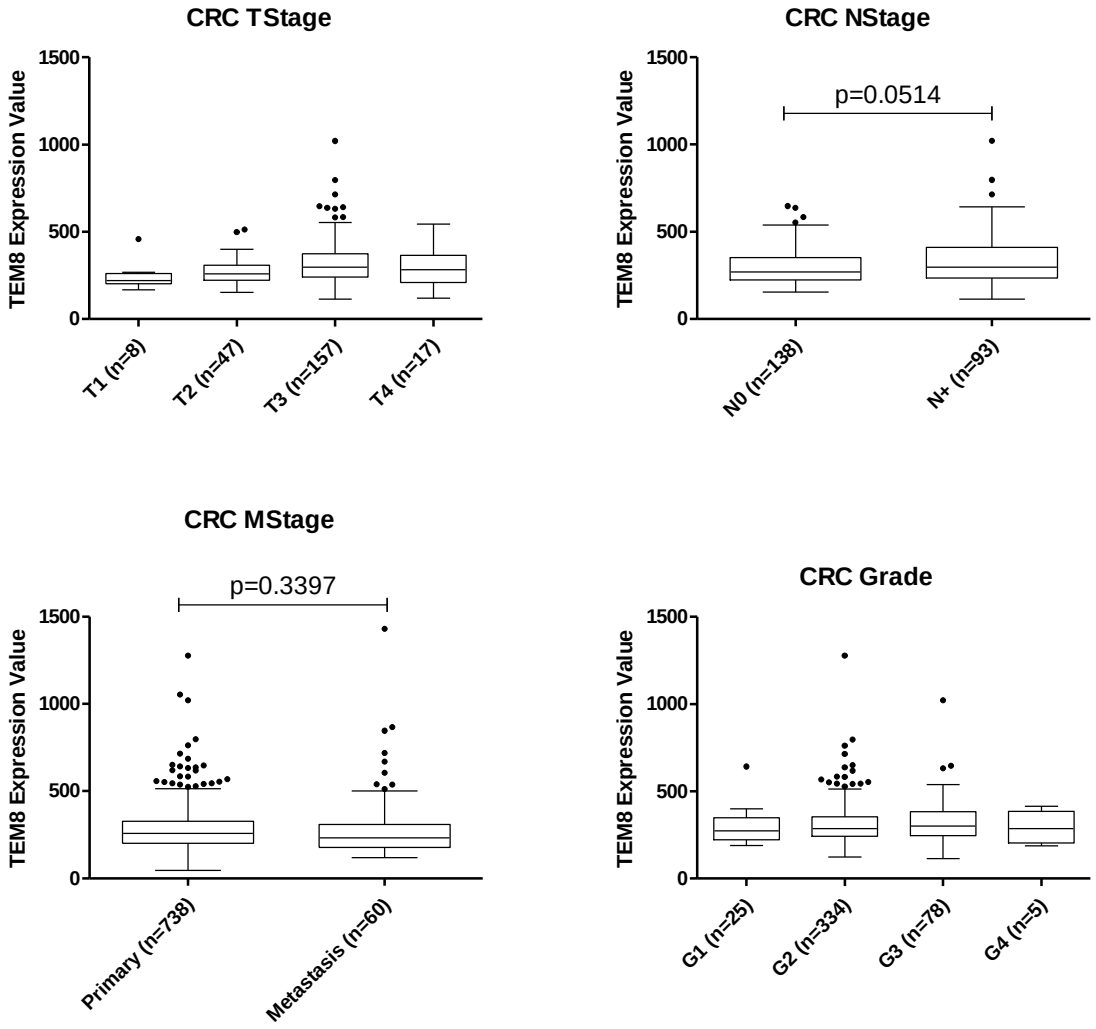

Figure 24 | TEM8 Expression in Colorectal Cancer (CRC) Patient Samples

In silico transcrpitomic data were used to evaluate the relationship between TEM8 expression and various CRC clinico-pathological features. T Stage, Lymph Node (LN) Status, Metastasis and Tumor Grade were all assessed with respect to TEM8 expression. Clinical data was not available for all specimens hence the disparity in sample size. T Stage and Grade were statistically evaluated using Kruskal-Wallis (T-Stage: $p=0.0143$, Grade: $p=0.3970$). Dunns multiple comparison post-test was used to assess significance with α set at $p<0.05$. No significant difference between T-stage groups even with Kruskal-Wallis $p<0.05$. Lymph Node Status and Metastasis were statistically evaluated using Mann Whitney non-parametric test.

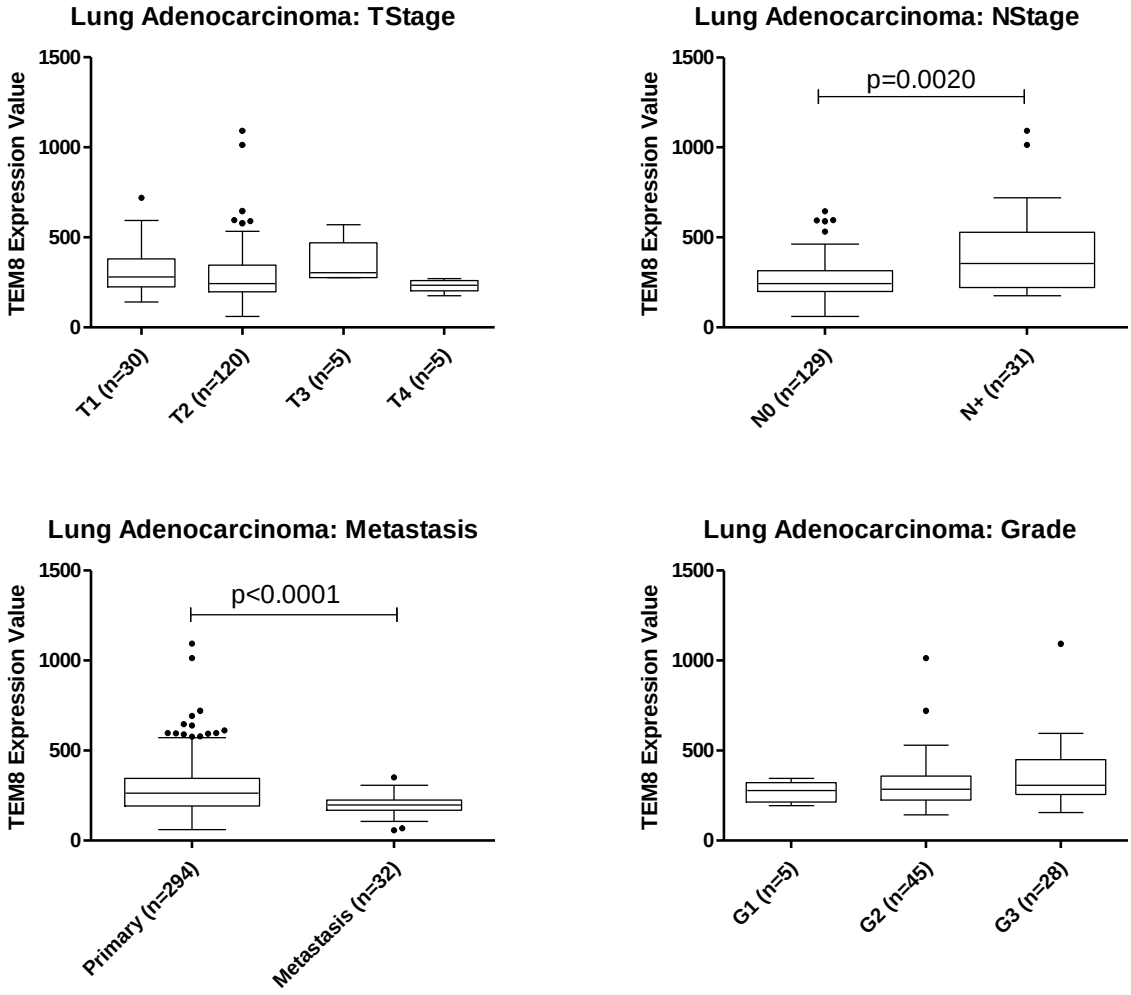

Figure 25 | TEM8 Expression in Lung Adenocarcinoma Patient Samples

In silico transcrpitomic data were used to evaluate the relationship between TEM8 expression and Lung Adenocarcinoma clinico-pathological features. T Stage, Lymph Node (LN) Status, Metastasis and Tumor Grade were all assessed with respect to TEM8 expression. Clinical data was not available for all specimens hence the disparity in sample size. T Stage and Grade were statistically evaluated using Kruskal-Wallis (T-Stage: p=0.1213, Grade: p=0.2092). Lymph Node Status and Metastasis were statistically evaluated using Mann Whitney non-parametric test.

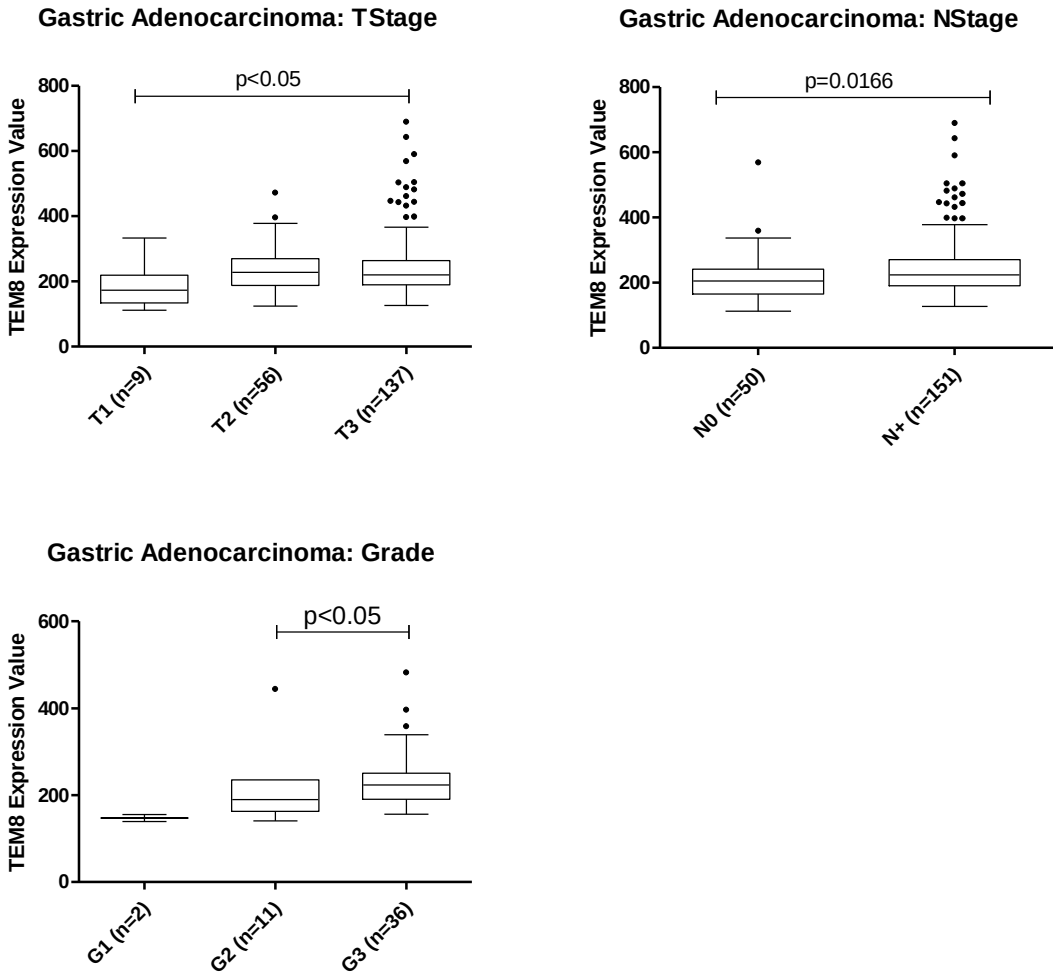

Figure 26 | TEM8 Expression in Gastric Adenocarcinoma Patient Samples

In silico transcrpitomic data were used to evaluate the relationship between TEM8 expression and Gastric Adenocarcinoma clinico-pathological features. T Stage, Lymph Node (LN) Postitivity, and Tumor Grade were all assessed with respect to TEM8 expression. Clinical data were not available for all specimens hence the disparity in sample size. Distant metastasis could not be evaluated. T Stage and Grade means were statistically different as per Kruskal-Wallis test (T-Stage: $p=0.0474$, Grade: $p=0.0136$). Dunns multiple comparison post-test was used to assess significance with α set at $p<0.05$. Lymph Node Status was statistically evaluated using Mann Whitney non-parametric test.