The tumour microenvironment: the effect of breast cancer cell conditioned medium on the endothelium

Thesis presented in partial fulfilment of the requirements for the degree Master of Science (Physiological Sciences) in the Faculty of Science at Stellenbosch University

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Declaration of originality

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Abstract

Background:

Breast cancer is the most common cancer diagnosed in women and the most common cancer globally. The human mammary gland is comprised of epithelium and vascular rich stroma. It has been established that breast cancer cells interact with and alter their stroma and neighbouring cells, to establish a tumour microenvironment (TME). Mammary endothelial cells are key targets to be transformed into tumour endothelial cells (TECs). These cells are genetically and phenotypically distinct from their normal, healthy counterparts and provide various pro-tumourigenic effects. These effects are modulated by the expression of various molecules that have been classified as TEC markers based on their expression in TECs compared to normal endothelial cells. As central role players in angiogenesis, TECs are key to tumour angiogenesis. Anti-angiogenic agents have proven to be effective, yet act as a double-edged sword, as a result of downstream complications and side effects. TECs therefore serve as potential targets for therapeutic intervention. Various role players in the tumour microenvironment have been investigated, but the effect of breast cancer cells on the tumour endothelial phenotype is not well established. The aims of this study were to evaluate a TEC phenotype in breast cancer and investigate how breast cancer impacts angiogenesis.

Methods:

Conditioned medium (CM) was harvested from non-malignant (MCF-12A) breast epithelial cells and from malignant (MCF-7 and MDA-MB-231) breast cancer cells starved of supplements and growth factors for 24 hours. Endothelial cells (HUVECs) were then treated with CM for 24 hours. To evaluate a TEC phenotype in breast cancer, cell viability (WST-1 assay), cell morphology (phase contrast imaging), and gene (reverse transcriptase-quantitative polymerase chain reaction) and protein (Western blots) expression of markers associated with a TEC phenotype were assessed. To assess angiogenesis in breast cancer, cell migration (scratch assay) and tube formation (tube formation assay) assays were utilised. A comparative model of non-malignant versus malignant signalling was used throughout the study.

Results:

Breast cell CM significantly increased HUVEC cell viability in all treatment groups. Changes in morphology were observed, which included elongation and branching, and occurred to a greater degree in malignant CM groups. TEC markers were significantly upregulated in response to non-malignant signalling and tumour endothelial marker 8 was observed to contribute to the TEC phenotype in breast cancer. Significant changes in cell migration were observed in the MCF-7 CM

group. Furthermore, clear qualitative differences in the tube formation of HUVECs were noted in malignant groups compared to the non-malignant group.

Conclusion:

Our results highlight the fact that endothelial cells are highly responsive to interactions with nutrient deprived breast cells but the interaction with non-malignant breast cells compared to malignant breast cells is significantly different. Breast cancer cells therefore do alter endothelial cells, but the characteristic TEC phenotype is not specific to a malignant response. Breast cancer cells alter the angiogenic process but the degree of hyperactivation is influenced by the breast cancer phenotype. It is therefore evident that endothelial cells and angiogenesis are altered and key to breast cancer progression, yet a TEC phenotype specific to breast cancer remains to be defined.

Opsomming

Inleiding:

Borskanker is die mees algemene tipe kanker wat by vroue gediagnoseer word en is ook die algemeenste kanker wêreldwyd. Die menslike melkklier bestaan uit epiteel en vaskulêre ryk stroma. Dit is vasgestel dat borskankerselle in wisselwerking tree met en hul stroma en naburige selle verander om 'n tumormikro-omgewing (TME) te vestig. Bors endoteelselle word gereeld geteiken om in tumor endoteelselle (TECs) omskep te word. Hierdie selle is geneties en fenotipies onderskei van hul normale, gesonde eweknieë en verskaf verskeie pro-tumorigeniese effekte. Hierdie effekte word gemoduleer deur die uitdrukking van verskeie molekules wat as TEC-merkers geklassifiseer is, gebasseer op hul uitdrukking in TECs in vergelyking met normale endoteelselle. As sentrale rolspelers in angiogenese, speel TECs ‘n belagrike rol in tumor angiogenese. Dit is al bewys dat anti-angiogene middels effektief is, maar dien steeds as 'n tweesnydende swaard, as gevolg van stroomaf komplikasies en newe-effekte. TECs dien dus as potensiële teikens vir terapeutiese intervensie. Verskeie rolspelers in die tumor mikro-omgewing is ondersoek, maar die effek van borskankerselle op die tumor endoteel fenotipe is nie goed gevestig nie. Die doel van hierdie studie was om 'n TEC-fenotipe in borskanker te evalueer en om te ondersoek hoe borskanker angiogenese beïnvloed.

Metodes:

Gekondisioneerde medium (CM) is geoes van nie-kwaadaardige (MCF-12A) borsepiteelselle en van kwaadaardige (MCF-7 en MDA-MB-231) borskankerselle wat vir 24 uur lank van aanvullings en groeifaktore uitgehonger is. Endoteelselle (HUVECs) is daarna vir 24 uur met borssel CM behandel en aan ontledings onderwerp. Om 'n TEC-fenotipe in borskanker te vestig, is sellewensvatbaarheid (WST-1-toets), selmorfologie (fasekontrasbeelding), en geen- (omgekeerde transkriptase-kwantitatiewe PCR) en proteïen (Western blots) uitdrukking van merkers geassosieer met 'n TEC-fenotipe, geëvalueer. Om angiogenese in borskanker te bepaal, is selmigrasie (krap-toets) en buisvorming (buisvorming-toets) gebruik. 'n Vergelykende model van nie-kwaadaardige versus kwaadaardige seinoordrag is deur die hele studie gebruik.

Resultate:

Borssel-CM het HUVEC-sellewensvatbaarheid in alle behandelingsgroepe aansienlik verhoog. Veranderinge in morfologie is waargeneem, wat verlenging en vertakking ingesluit het, en het tot 'n groter mate in kwaadaardige CM-groepe voorgekom. TEC merkers is aansienlik opgereguleer in reaksie op nie-kwaadaardige sein en tumor endoteel merker 8 is waargeneem om by te dra tot die

TEC fenotipe in borskanker. Beduidende veranderinge in selmigrasie is waargeneem in die MCF-7 CM groep. Verder is duidelike kwalitatiewe verskille in die buisvorming van HUVECs opgemerk in kwaadaardige groepe in vergelyking met die nie-kwaadaardige groep.

Gevolgtrekking:

Ons resultate beklemtoon die feit dat endoteelselle sterk reageer op interaksies met borselle waarvan die voedingstowwe ontneem is, maar die interaksie met nie-kwaadaardige borselle in vergelyking met kwaadaardige borselle is aansienlik anders. Borskankerselle verander dus endoteelselle, maar die kenmerkende TEC-fenotipe is nie spesifiek vir 'n kwaadaardige fenotipe nie. Borskankerselle verander die angiogeniese proses, maar die mate van hiperaktivering word deur die borskankerfenotipe beïnvloed. Dit is dus duidelik dat endoteelselle en angiogenese verander is en die sleutel tot borskanker bevordering is, maar 'n TEC-fenotipe wat spesifiek vir borskanker is, moet nog gedefinieer word.

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Conferences

Stellenbosch University Faculty of Science Postgraduate Research Conference – 1st annual conference

Presentation title: The tumour microenvironment: the effect of breast cancer on the endothelium

Physiology Society of Southern Africa (PSSA) Conference – 45th annual Conference

Presentation title: The tumour microenvironment: the effect of breast cancer on the endothelium

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Abbreviations

Symbol:

$2^{-\Delta\Delta Ct}$	–	Delta delta quantitation

A

ANOVA	–	Analysis of variance
ANTXR1	–	Anthrax toxin receptor 1

B

bFGF	–	Basic fibroblast growth factor
BSA	–	Bovine serum albumin

C

CAF	–	Cancer-associated fibroblasts
CM	–	Conditioned medium
CXCL	–	Chemokine CXC ligand
CXCR	–	Chemokine CXC receptor
CTC	–	Circulating tumour cells

D

Dll4	–	Delta-like ligand 4
DMEM	–	Dulbecco's Modified Eagle Medium
DNA	–	Deoxyribonucleic acid

E

ECM	–	Extracellular matrix

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

eNOS – Endothelial nitric oxide synthase

ER – Estrogen receptor-α

EV – Extracellular vesicles

F

FBS – Fetal Bovine Serum

FGF – Fibroblast growth factor

J

JAK2/STAT3 – Janus kinase 2/signal transducer and activator of transcription 3

H

HER-2 – Human epidermal growth factor receptor-2

HIF – Hypoxia inducible factor

HUVECs – Human umbilical vein endothelial cells

I

IA – Intussusceptive angiogenesis

IDT – Integrated DNA Technologies

L

LOX – Lysyl oxidase

M

MAPK – Mitogen activated protein kinase

MCM2 – Mini-chromosome maintence-2

MDR1 – Multidrug resistance 1

MMC – Mitomycin C

mRNA – Messenger RNA

MT1-MMP – Membrane type 1-matrix metalloproteinase

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole

N

NBF – Neutral Buffered Formalin

NLPR3 – NLR-family pyrin domain-containing protein 3

NO – Nitric oxide

P

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PDGF – Platelet derived growth factor

Penstrep – Penicillin-Streptomycin

PI3K/Akt – Phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt)

PR – Progesterone receptor

R

RIPA – Radioimmunoprecipitation assay

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT-qPCR – Reverse transcriptase quantitative real time polymerase chain reaction

S

SA – Sprouting angiogenesis

SDS-PAGE – Sodium dodecyl-sulphate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

T

TBS-T – Tris-Buffered Saline-Tween® 20

TEC – Tumour endothelial cell

TEM – Tumour endothelial marker

TGF – Transforming growth factor

TLR – Toll like receptor

TME – Tumour microenvironment

TNBC – Triple negative breast cancer

V

vSMCs – Vascular smooth muscle cells

VEGF – Vascular endothelial growth factor

VEGFR – Vascular endothelial growth factor receptor

W

WST-1 – Water-soluble tetrazolium salt-1

Units of Measurement

°C	–	Degree Celsius
µg	–	Microgram
µg/µL	–	Microgram per microlitre
µg/mL	–	Microgram per millilitre
µL	–	Microlitre
µM	–	Micromolar
Bp	–	Base pairs
G	–	G-force
g/mL	–	Grams per millilitre
kDa	–	Kilodalton
L	–	Litre
mg/mL	–	Milligram per millilitre
mL	–	Millilitre
mm	–	Millimetre
mM	–	Millimolar
ng/mL	–	Nanogram per millilitre
nm	–	Nanometre
RPM	–	Revolutions per minute
V	–	Volt

Chapter 1: Literature review

1.1 An introduction to breast cancer

Breast cancer has become the most frequently diagnosed cancer globally since the end of 2020. This revelation was met with a global diagnosis of 2.3 million cases and 685 000 deaths (World Health Organization, 2021). According to the latest report from the National Cancer Registry (South Africa), breast cancer had the highest incidence rate among women, accounting for 23% of all cases (CANSA, 2022; National Health Laboratory Service, 2019). Furthermore, the disease burden of breast cancer is also greater than that of any other cancer (World Health Organization, 2021; Ataollahi et al., 2015). In South Africa, the risk of developing breast cancer was estimated to be 1 in 26 women (CANSA, 2022; National Health Laboratory Service, 2019).

Cancer is defined as abnormal cell growth that may occur in various types of cells located throughout the body (Cooper, 2000). A set of characteristics that classify the malignant phenotype were identified by Hanahan & Weinberg (2011), titled the hallmarks of cancer. Among these hallmarks are sustained proliferative signalling, resistance to cell death, invasion and metastasis, and inducing angiogenesis. They serve as prognostic factors in cancer diagnoses and staging, and impact therapeutic intervention (Place, Huh & Polyak, 2011). Yet, despite the vast improvements that have been made in treatment and prevention, there remains an urgent need to further understand breast cancer and to improve a patient's quality of life.

1.1.1 Breast cancer classification

Breast cancer classification is complex because of the range of genetic subtypes. It encompasses the size of the tumour, lymph node involvement and metastatic potential (TNM staging); histological type; receptor status; and the presentation of biomarkers (Bagaria et al., 2014; Sinn & Kreipe, 2013; Pusztai et al., 2006). Emphasis is placed on receptor status because it is a good indicator of prognosis and treatment responsiveness and outcomes (Vuong et al., 2012). Receptor status is based on the presence of the estrogen receptor-α (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2).

Estrogens are the main sex steroid hormones in the mammary gland, with a wide range of roles including morphogenesis, ductal formation, and gene activation (Feng et al., 2007; Mallepell et al., 2005; Nilsson & Gustafsson, 2011). In breast cancer, the ERs are the main drivers of carcinogenesis, implicated in tumourigenic activities such as proliferation and metastasis (Roy & Vadlamudi, 2012; Stingl, 2011). Progesterones are sex steroid hormones that are regulated by estrogens and are

involved in mammary gland development & growth, ductal formation, and branching (Ruan, Monaco & Kleinberg, 2005; Brisken et al., 1998). PRs are also major drivers of tumour growth and are implicated in tumourigenic activities including proliferation and tumourigenic gene transcription (Scabia et al., 2022; Daniel, Hagan & Lange, 2011). HER-2s are membrane receptors expressed at low levels and activated through (1) heterodimerization with other ligand-bound epidermal growth factor receptor (EGFR) family members or (2) homodimerization as a result of being constitutively activated. HER-2 activation contributes to various processes, including cell differentiation, cell proliferation, cell survival, cell migration and angiogenesis (Furrer et al., 2018; Iqbal & Iqbal, 2014; Hynes & Watson, 2010; Graus-Porta et al., 1997). HER-2 positive breast cancer is characterized by HER-2 overexpression. It therefore contributes to the aberrant activation and dysregulation of downstream pathways and is associated with a worse prognosis (Furrer et al., 2018; Slamon et al., 2001; Pegram, Konecny & Siamon, 2000).

Four types of breast cancer have been classified based on receptor status. ER and PR receptor positive breast cancers are classified as luminal A and B based on HER-2 overexpression. ER and PR negative breast cancers overexpressing HER-2 are classified as non-luminal, and breast cancers lacking ER, PR and HER-2 receptors are classified as Triple negative, as illustrated in **Figure 1.1**. Breast cancer subtypes are identified using immunohistochemistry and gene-based assays (Gao & Swain, 2018).

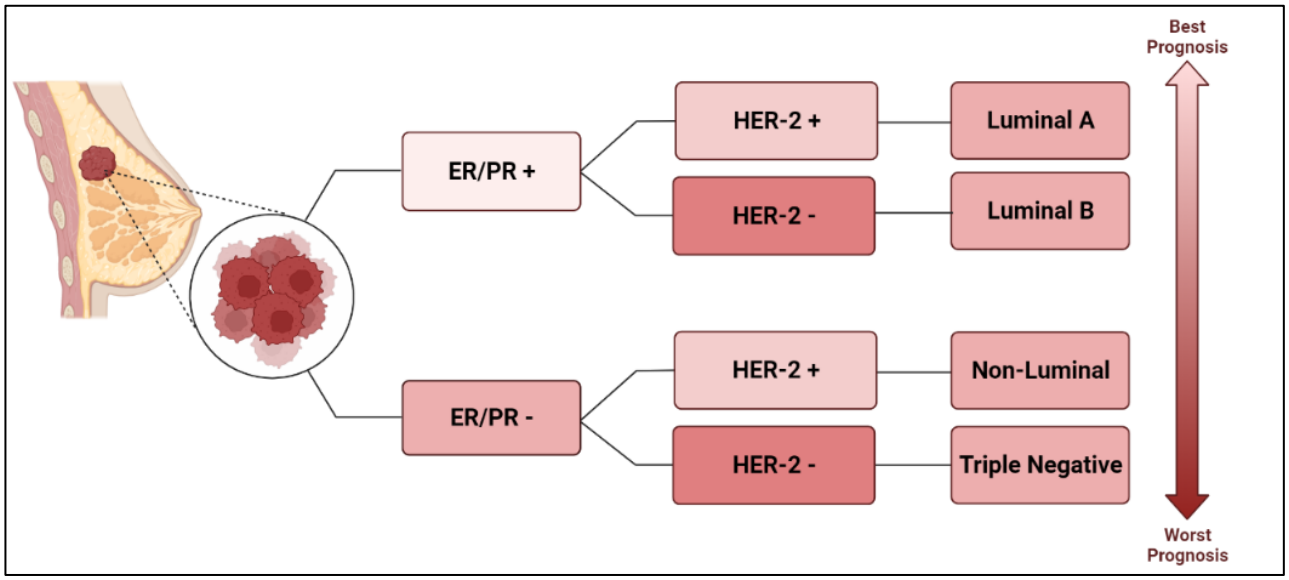

Figure 1.1: Hormone receptor expression-based breast cancer subtypes. Abbreviations: ER, Estrogen receptor-α; HER-2, Human epidermal growth factor receptor-2; PR, Progesterone receptor (Created in Biorender.com).

The most aggressive form of breast cancer is triple negative breast cancer (TNBC), an advanced stage invasive breast cancer that is highly metastatic and often associated with poorer outcomes. It accounts for 10-20% of breast cancers and patients with TNBC generally have decreased survival rates compared to non-TNBC breast cancer patients (Kennecke et al., 2010; Kondov et al., 2018; Lanning et al., 2017). TNBCs can be classified as a range of subtypes, including basal-like and normal-like cancers, but the most distinctive feature is the absence of hormone and growth factor receptors (Yersal & Barutca, 2014). Furthermore, various cell components in the TNBC environment can be used as prognostic factors to guide the treatment of TNBC as this cancer is often characterized by a highly integrated stromal environment. Several cell types such as fibroblasts, macrophages and adipocytes play an important role in breast cancer development and progression.

As a result of the variety of cell types present within the mammary gland and a great array of breast-cancer causing mutations, breast cancer encompasses several distinct malignancies of the mammary gland. The architecture of the mammary gland is also significantly altered during breast cancer. Instead of bilayers, there are several layers composed of cells with increased proliferative rates, partially lacking cell polarity with less intercellular adhesions (Ewald et al., 2008; Bilder, 2004). It has therefore become evident that the stroma, which constitutes the microenvironment, plays a crucial role in cancer progression and treatment response (Place, Huh & Polyak, 2011).

1.1.2 The Mammary Gland

The human mammary gland is a compound, branched tubuloaveloar gland primarily responsible for milk secretion. It is comprised of two compartments: the epithelium and the surrounding vascular rich stroma, which are separated by a basement membrane (Biswas et al., 2022; Khan & Sajjad, 2021). The epithelium forms the bi-layered terminal duct lobular units, giving rise to a highly branched epithelial tube network. The epithelium is divided into the inner luminal epithelial cell layer, which is characterized by its hormone receptor status and form secretory alveoli, and the outer basal epithelial layer, which is comprised of myoepithelial and stem cells (Feng et al., 2018; Macias & Hinck, 2012; Place, Huh & Polyak, 2011; Ewald et al., 2008). These cells are influenced by various cycles, which modulate proliferation, branching/structure and regression depending on the hormonal balance. The organs that modulate mammary gland activity include the pituitary and adrenal gland (hypothalamic-pituitary-adrenal axis), ovaries, uterus and liver (Brisken & O'Malley, 2010; Macias & Hinck, 2012; Seachrist et al., 2018; Strange et al., 2007).

As previously mentioned, the stroma is a supportive compartment comprised of fibroblasts, immune cells, endothelial cells, adipocytes (mammary fat pad), nerves, and the extracellular matrix (ECM), constituting the bulk of the mammary gland (Dawson & Visvader, 2021; Ingthorsson et al., 2010).

The stroma is therefore important for various reasons, including the physical support, the instructive cues and regulatory signalling for development, patterning and function, and the development and progression of cancer in some cases (Sternlicht, 2005; Wiseman & Werb, 2002).

The life cycle of the mammary gland is physiologically dictated, encompassing immense changes in cell composition, cell number, structure and cell function throughout the hormone-independent stages of embryogenesis and puberty, and the hormone-dependent stages of pregnancy, lactation, and involution (Brisken & O'Malley, 2010; Djonov, Andres & Ziemiecki, 2001; McGee et al., 2006). Cell differentiation and growth in the mammary glands are regulated by local and systemic signalling, hormone activity (ER, PR and HER-2), and epithelial-stromal interactions (McGee et al., 2006; Dulbecco, Henahan & Armstrong, 1982). There are two major cycles that dictate the hormonal regulation of mammary epithelium: the menstrual cycle and pregnancy. Cell response to the hormone stimulation includes proliferation, differentiation and cell death, however, the response is regional and only affects subsets of the epithelial population (Andres & Strange, 1999).

The three-dimensional structures that comprise the epithelial tube network are largely influenced by cell polarization, specific cell-cell contacts, attachment to the underlying basement membrane, as well as mechanical forces of and signalling from stromal cells. Epithelial-stromal interactions in the mammary gland are thus key to tissue homeostasis and function (McGee et al., 2006). Furthermore, vascularization in the mammary gland is significant to compensate for the massive changes that necessitate variable oxygenation and nutrient delivery, where blood vessels mainly wrap around and line the mammary ducts. Along with their general functions, endothelial cells also need to provide "angiogenesis on demand" accompanied by "controlled vascular regression" (Dawson & Visvader, 2021; Djonov, Andres & Ziemiecki, 2001). The endothelial cells are generally in a state of quiescence yet become highly active during pregnancy and lactation. These cells undergo angiogenesis throughout, starting with sprouting angiogenesis at the beginning of pregnancy – to deal with epithelial expansion, then switching to intussusceptive angiogenesis until post lactational involution of the mammary gland to return to a resting state – terms that will be elaborated on later. Endothelial cells are however not hormonally regulated and respond to different cues (Dawson & Visvader, 2021; Djonov, Andres & Ziemiecki, 2001; Matsumoto et al., 1992; Watson, 2006),

1.2 An introduction to endothelial cells

Endothelial cells are specialized cells that line the inner surface of blood vessels of the entire cardiovascular system. Blood vessels serve as the barrier between blood and tissues. They are also the highly regulated route of passage for fluids, molecules, and cells (Yazdani et al., 2019). Blood vessels are comprised of three layers namely, (1) the tunica adventitia, the outermost layer, which

is comprised of collagen and elastic fibres; (2) the tunica media, the intermediate/middle layer, which is comprised of collagen, elastin and smooth muscle cells; and (3) the tunica intima, the innermost layer, which is comprised of the endothelial cells that are exposed to blood in the vessel lumen. The two outer layers are specialized for function, shape, support and stability during vessel maturation, while the tunica intima serves as the general component of all blood vessels (Tucker et al., 2021; Betz et al., 2016). Blood vessels are classified in the order of arteries, veins, arterioles, venules, and capillaries. Capillaries are the thinnest or smallest form of a vessel, comprised of an endothelial layer, basement membrane, and adventitia. They are the point of contact between blood and tissues and possess various tissue-specific characteristics, including the degree of permeability and endothelial coverage (continuous vs non-continuous) (Krüger-Genge et al., 2019; Palade, 1961).

The endothelium in its entirety is regarded as an organ that carries out endocrine, autocrine and paracrine functions, yet these cells are structurally and functionally heterogenous. The heterogeneity is influenced by “location” which influences the type of endothelial cell (arterial vs vein, large vessel vs capillary, etc,), as well as tissue-specific requirements (Marcu et al., 2018; Grochot-Przęczek et al., 2013; Galley & Webster, 2004). Endothelial cells exist in a monolayer and are anchored to the basement membrane via focal adhesions. Differences in structure or phenotype include tissue-specific cell-coupling junctions, cell shape and size, the permeability of the capillary bed and the degree of mural cell coverage (Marcu et al., 2018; Félétou, 2011; Red-Horse et al., 2007; Simonescu & Simonescu, 1988).

The functions of endothelial cells are also vast, ranging from general to specialized, including but not limited to: (1) formation of a selective barrier between tissues and blood, which is crucial for oxygen (oxygen sensors) and nutrient delivery, as well as the removal of metabolic waste; (2) vascular tone regulation, blood flow and blood fluidity; (3) maintaining the balance between coagulation and fibrinolysis; (4) organ development by providing paracrine signals over short distances, referred to as inductive signals, and molecules involved in patterning that are required for cell guidance; (5) stem cell maintenance and organ regeneration and (6) functioning in innate and adaptive immune responses and the regulation of inflammation (Reiterer & Branco, 2020; Krüger-Genge et al., 2019; Rafii, Butler & Ding, 2016; Sigurdsson et al., 2011; Red-Horse et al., 2007; Michiels, 2004; Lustig & Kirschner, 1995). The variety of activities provided and modulated by these cells are attributed to both a resting and active state, which stresses their importance in bodily and organ functioning to maintain homeostasis (Reiterer & Branco, 2020; Serratì et al., 2008). However, these cells are often subject to dysfunction and the disruption of cardiovascular homeostasis serves as a predecessor of various disease processes and consequences.

1.2.1 Endothelial dysfunction

The term endothelial dysfunction is a broad term and a complex phenomenon. At the core it describes endothelial activation (pathological), encompassing a host defence response and several maladaptive changes in functional phenotype (Fountoulakis et al., 2017; Gimbrone & García-Cardeña, 2016; Deanfield, Halcox & Rabelink, 2007). It can be both a consequence of negative changes to cardiovascular health and a predecessor of cardiovascular conditions and diseases, such as hypertension and atherosclerosis (van der Velde, Meijers & de Boer, 2015; Bonetti, Lerman & Lerman, 2003).

A key characteristic of healthy endothelial cells is the ability to secrete nitric oxide (NO). It is a compound through which these cells modulate their regulatory functions, produced by the endothelial NO synthase (eNOS) enzyme. However, during endothelial dysfunction, there is an increased generation of reactive oxygen species (ROS) which not only leads to oxidative stress but also the reduced bioavailability of NO through the uncoupling of eNOS by pro-oxidant radicals (Feng & Hedner, 1990; Halcox, 2012). In the activation of these cells, various signalling pathways are activated, including the NLR-family pyrin domain-containing protein 3 (NLRP3) inflammasome and the mitogen activated protein kinase (MAPK) pathway (Bai et al., 2020; Sun et al., 2020). This leads to a wide range of changes, including the disruption of the redox balance, inflammation (acute and chronic), thrombosis and vasoconstriction; pathological states which are accompanied by increased cell adhesion and the disruption of the endothelial barrier permeability (Gimbrone & García-Cardeña, 2016; Flammer & Lüscher, 2010)

Endothelial ‘health’ is central to cardiovascular health. Endothelial dysfunction is predominantly studied and portrayed as the precursor to morphological atherosclerotic changes and the development of lesions, which later manifests as clinal cardiovascular complications (Deanfield, Halcox & Rabelink, 2007). Links have also been established between cardiovascular health and downstream cancer development. Poor cardiovascular health, ranging from endothelial dysfunction to myocardial infarction, increases the risk of cancer, tumour growth, risk of reoccurrence and the chance of cancer-related death (Koelwyn et al., 2020; Toya et al., 2020; Franses et al., 2013). These effects are mediated through pro-inflammatory signalling and immunosuppressive activity (Koelwyn et al., 2020; Franses et al., 2013). As important as endothelial cells are to cardiovascular health, they are important to tissue vascularization and functioning, which is achieved through angiogenesis.

1.3 Angiogenesis

Angiogenesis refers to the formation of new blood vessels from existing vasculature (Cavallaro & Christofori, 2000). This is a normal biological process requiring an exact balance of stimulatory and inhibitory signals (Buchanan et al., 2012). It occurs throughout an organism's lifespan to provide developing, reproductive, and healing tissues with nutrients and oxygen, however, it is downregulated in adulthood. Endothelial cells lining adult vasculature are thus quiescent, yet still maintain a high level of plasticity (Potente, Gerhardt & Carmeliet, 2011; Papetti & Herman, 2002).

Angiogenesis is often widely used to refer to all forms of blood vessel formation or neovascularization. Yet clinically, it is classified as a type of neovascularization, along with arteriogenesis and vasculogenesis. Arteriogenesis is blood vessel remodelling or *de novo* formation of arteries; vasculogenesis is blood vessel remodelling or *de novo* formation with circulating vascular precursor cells; while angiogenesis is the formation of capillaries from pre-existing post-capillary venules (from the base of the capillary bed) (Simons, 2005; Carmeliet, 2003; Polverini, 1995; Mahadevan & Hart, 1990). As previously mentioned, there are two types of angiogenesis – sprouting angiogenesis (SA) and intussusceptive angiogenesis (IA) which are two distinct processes triggered under different conditions.

1.3.1 Sprouting angiogenesis (SA)

SA is vascular growth triggered when oxygen-sensing mechanisms necessitate an angiogenic response in conditions of poor perfusion (Adair & Montani, 2010). Tissue hypoxia is, therefore, the main trigger for angiogenesis. During hypoxia, hypoxia inducible factor-1α (HIF-1α), which is degraded under normoxic conditions, and HIF-1β subunits dimerize. This produces HIF, which acts as a transcription factor that binds to hypoxia responsive elements on hypoxia-inducible genes that mediate various processes including angiogenesis (Huang et al., 1998; Semenza et al., 1991; Wang et al., 1995)

This process is mediated by a wide range of soluble factors that serve as angiogenic inducers and promotors or inhibitors; cell-cell and cell-matrix interactions mediated by membrane bound molecules; and the mechanical forces that constitute hemodynamics (Papetti & Herman, 2002). Growth factors are responsible for the angiogenic process and various growth factors serve as angiogenic inducers. These angiogenic growth factors initiate an angiogenic response by activating a signalling cascade that promotes the migration, proliferation, and survival of endothelial cells after binding to receptors expressed on endothelial cells (Fitzgerald, Soro-Arnaiz & de Bock, 2018; Matsuo et al., 2009).

The main coordinators of SA are vascular endothelial growth factor (VEGF)-A and platelet derived growth factor (PDGF)-B. VEGF-A plays a role in endothelial stimulation (physiological activation), blood vessel destabilization, and endothelial migration and proliferation; while PDGF-B mediates vessel maturation and stabilization through the recruitment of mural cells, such as vascular smooth muscle cells (vSMCs) and pericytes (Shibuya, 2011; Raica & Cimpean, 2010; Stuttfeld & Ballmer-Hofer, 2009; Gerhardt et al., 2003; Lindahl et al., 1999). VEGF-A, its receptors VEGF Receptor 1 (VEGFR1) & VEGFR2 and its co-receptors Neuropolin-1 and -2, are the main and most potent mediators of both physiological and pathophysiological angiogenesis. Although mainly increased in response to HIF activity, additional factors perpetuate VEGF activity, including the paracrine release of VEGF by various neighbouring cell types, including pericytes. The paracrine and autocrine release of certain growth factors and cytokines, including transforming growth factor (TGF)-α & -β, PDGFs & epidermal growth factor (EGF), also stimulate VEGF expression while having their own angiogenic influence (Stuttfeld & Ballmer-Hofer, 2009; Ferrara, Gerber & LeCouter, 2003; Reynolds, Grazul-Bilska & Redmer, 2000; Neufeld et al., 1999).

SA is a slow and sequential process that can be divided into four phases. The first phase: endothelial stimulation and vessel destabilization, encompasses tip cell selection, stalk cell selection, metalloproteinase secretion and vessel destabilization. The second phase: sprouting and branching, encompasses endothelial migration and proliferation. The third phase: lumen formation and anastomosis, encompasses lumen formation and expansion, and vascular anastomosis of growing sprouts. The fourth phase: vessel maturation and stabilization, encompasses functional lumen formation, endothelial quiescence, mural cell recruitment, blood flow initiation, and vessel pruning, as depicted in **Figure 1.2**.

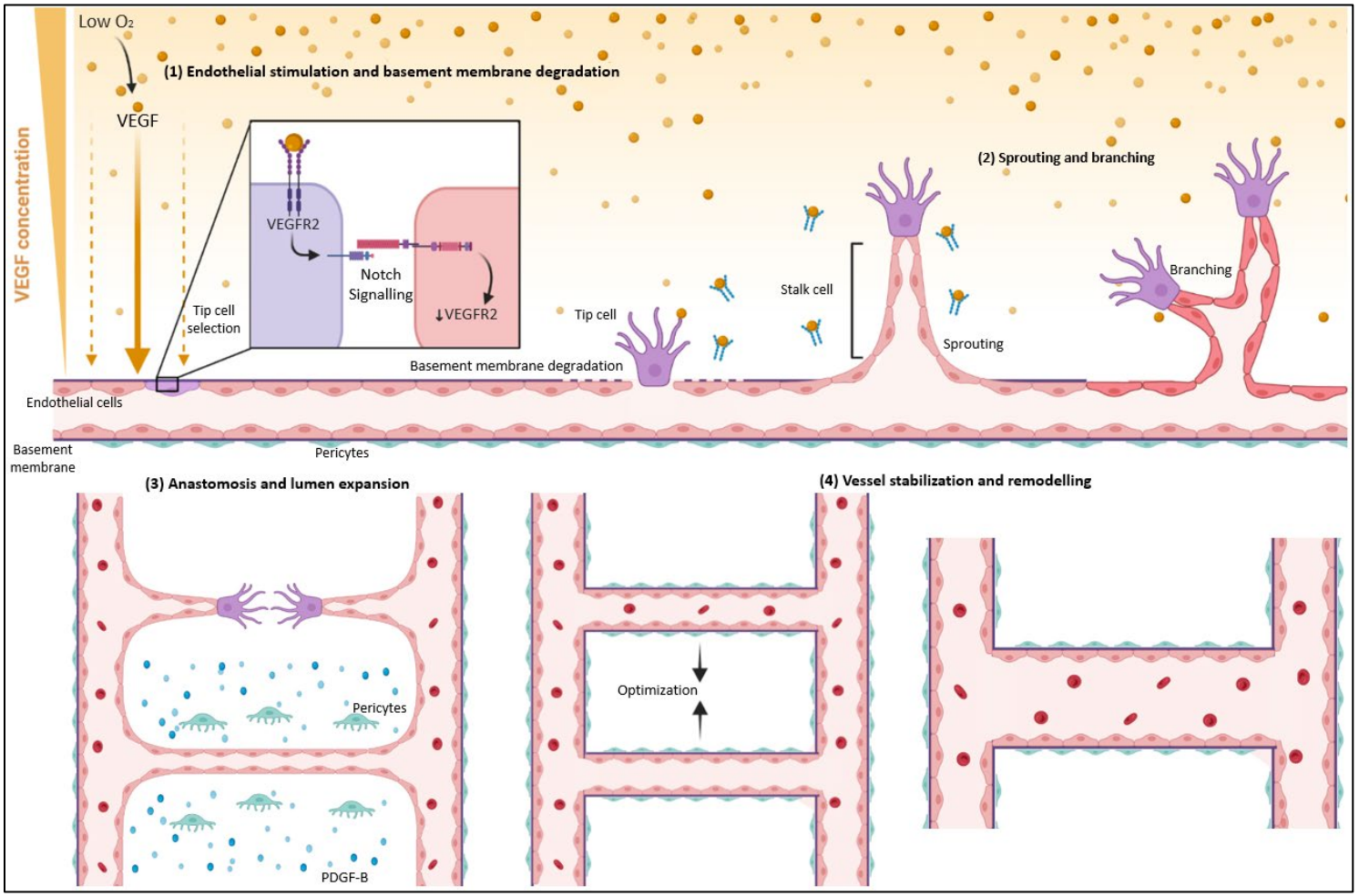

Figure 1.2: A brief overview of sprouting angiogenesis. Sprouting angiogenesis is a tightly controlled process that can be divided into four phases: (1) Endothelial stimulation and basement membrane degradation; (2) Sprouting and branching; (3) Anastomosis and lumen expansion; and (4) Vessel stabilization and remodelling. VEGF is the main angiogenic mediator released under conditions of low oxygen (hypoxia) and is responsible for blood vessel formation, while PDGF-B is involved in blood vessel stabilization, which involves pericyte recruitment and basement membrane generation. Blood flow is subsequently initiated, and the vessel network is optimized. Abbreviations: PDGF-B, Platelet derived growth factor-B; VEGF, Vascular endothelial growth factor; VEGFR2, Vascular endothelial growth factor receptor 2 (Created in Biorender.com).

Tip cells are the cells that guide the newly forming sprout and are selected upon stimulation by pro-angiogenic growth factors (Potente, Gerhardt & Carmeliet, 2011; Gerhardt et al., 2003). These cells are characterized by numerous protruding filopodia that allow attachment to the ECM and stress fibre contraction that allows forward progression. These cells are thus highly motile to allow exploration of the microenvironment - their phenotype being migratory but not proliferative (Gerhardt et al., 2003; Papetti & Herman, 2002). Vessels are destabilized by proteolytic breakdown and remodelling of the ECM and basement membrane, hyperpermeability and pericyte detachment (Helfrich & Schadendorf, 2011; Bergers & Benjamin, 2003; Craft & Harris, 1994).

Once the tip cell is selected, it signals neighbouring endothelial cells to assume the fate of a stalk cell via VEGFR-Delta-like ligand 4 (DII4)-Notch signalling. A stalk cell phenotype allows sprout extension, continued connection to the parent vessel and the formation of a new vessel, with a phenotype that is proliferative and not migratory (Potente, Gerhardt & Carmeliet, 2011; Jakobsson et al., 2010). These cell phenotypes are, however, dynamic as cells ‘meet new neighbours’ and signalling dynamics change (Jakobsson et al., 2010).

Tip cells migrate according to chemotactic signals or paths with the least physical hindrance, while stalk cells proliferate and elongate. Tip cells migrate according to a gradient of VEGF, while stalk cells proliferate according to its concentration (Schuermann, Helker & Herzog, 2014; Gerhardt et al., 2003). As the sprouts ‘grow’, (1) the parent vessel’s lumen is connected to the newly forming sprout resulting in lumen formation, and (2) the tip cells will either encounter another sprout’s tip cell or a pre-existing functioning blood vessel, leading to vascular anastomosis (Sauteur et al., 2014; Schuermann, Helker & Herzog, 2014).

Lumen formation can be a complex process and involves extracellular and intracellular mechanisms of action. Extracellular processes are cord hollowing, lumen ensheathment and budding; while the intracellular processes are cell membrane invagination and cell hollowing (Schuermann, Helker & Herzog, 2014). Vascular anastomosis encompasses endothelial recognition, cell polarization and membrane invagination, leading to unicellular tube formation, and subsequent cell reorganisation and splitting, which lead to multicellular tube formation. It is an essential and characteristic process preceding the formation of functional lumens and vascular loops to establish a new vascular network to initiate blood flow, while being regulated by hemodynamic forces (Betz et al., 2016; Lenard et al., 2013).

The cessation of growth factor signalling and the flow of blood signal the activated endothelial cells to mature into quiescence. They are then referred to as phalanx cells and are responsible for the generation of the basement membrane, recruiting pericytes and smooth muscle cells, and the formation

of tight junctions (Potente, Gerhardt & Carmeliet, 2011; Bergers & Benjamin, 2003). Once functional, the network is remodelled by vascular pruning, optimizing for tissue perfusion and oxygen and nutrient delivery (Fitzgerald, Soro-Arnaiz & de Bock, 2018; Schuermann, Helker & Herzog, 2014). Vascular pruning is regulated by hemodynamic forces and considered the reverse of vascular anastomosis. In non-perfused vessels, the lumen collapses, cells reorganize resulting in a unicellular connection. In perfused vessels, cells undergo self-fusion, inducing cell reorganization, the formation of a unicellular tube and subsequent lumen collapse. Contact between the remaining cell and one of the vessels reduces and is subsequently diminished, completing the pruning process (Lenard et al., 2015; Schuermann, Helker & Herzog, 2014).

1.3.2 Intussusceptive angiogenesis (IA)

Intussusceptive or splitting angiogenesis (IA) is vascular remodelling in perfused tissues for the optimization of blood flow (Adair & Montani, 2010). It is therefore the reorganization and optimization of the vascular network, whereas SA, vasculogenesis and arteriogenesis are responsible for establishing the vascular network (de Spiegelaere et al., 2012). However, it was only discovered three decades ago and is not well understood. It is characterized by the formation of a distinctive intussusceptive-intraluminal pillar, splitting the existing blood vessels (Mentzer & Konerding, 2014; Paku et al., 2011). It differs from sprouting angiogenesis through the absence of vessel destabilization and sprouting (Egginton, Zhou & Hudlicka, 2001). The main trigger for intussusceptive angiogenesis is intraluminal hemodynamic forces, such as shear stress, hydrostatic pressure and flow fields induced by changes in blood flow. Although, it can also be affected by extraluminal influences, such as changes in metabolic needs, inflammation and growth factors (Mentzer & Konerding, 2014; de Spiegelaere et al., 2012; Egginton, Zhou & Hudlicka, 2001).

IA is executed by membrane type 1-matrix metalloproteinase (MT1-MMP) and its cleavage of thrombospondin-1 (Esteban et al., 2020). Downstream of MT1-MMP activity, the production of NO is triggered, leading to blood vessel dilation. Vasodilation is subsequently accompanied by an increase in blood flow and shear stress – triggering intussusceptive pillar formation and the generation of two new blood vessels – decreasing blood flow (D'Amico et al., 2020; Esteban et al., 2020). IA is best investigated in an adaptive response to increased blood flow in muscle and the pathological states of inflammatory bowel disease and tumour angiogenesis (D'Amico et al., 2020; Paku et al., 2011; Egginton, Zhou & Hudlicka, 2001).

IA can be divided into four phases. In the first phase multiple pillars are formed between opposite vessels in areas of low shear stress. In the second phase, the vessel wall is subsequently invaginated and the intraluminal pillars develop. The pillars mature and are reinforced by the recruitment of mural cells leading blood vessel splitting, in the third phase. In the fourth phase, the newly reorganized vascular network is optimized with vascular pruning (du Cheyne, Smeets & de Spiegelaere, 2021; Djonov et al., 2000).

Endothelial cells initially enlarge and flatten to form intraluminal bridges (Paku et al., 2011; Djonov et al., 2000). A small area of the basement membrane dissolves, and a bridge-forming endothelial cell joins a bundle of collagen in the supporting connective tissue. The endothelial actin cytoskeleton subsequently interacts with the collagen bundle through special attachment points. The collagen bundle is pulled in and transported through the vessel lumen, leading to the development and maturation of the intraluminal pillar as connective tissue cells immigrate and new collagenous connective tissue is deposited (du Cheyne, Smeets & de Spiegelaere, 2021; Paku et al., 2011). This prompts blood vessel splitting, which is adapted to intraluminal flow fields (Mentzer & Konerding, 2014; Burri, Hlushchuk & Djonov, 2004). Angiogenesis is therefore a highly co-ordinated and tightly controlled process, yet as is characteristic of tumours – processes and pathways are disrupted and altered – including angiogenesis through the establishment of a tumour microenvironment (TME).

1.4 An introduction to the tumour microenvironment (TME)

Tumours are considered as organs with the presence of a wide array of highly interactive cell types, mimicking normal tissues (Ziyad & Iruela-Arispe, 2011). Cancer cells interact (bi-directionally) with non-malignant stromal cells – hijacking & corrupting them to establish a ‘cancer-promoting’ microenvironment, referred to as the TME (Son et al., 2017; Ziyad & Iruela-Arispe, 2011; Wiseman & Werb, 2002). It can be defined as the surrounding tumourigenic niche of cellular and non-cellular components that allow the tumour to function as an ever-growing organ and to which distant non-malignant cells can also be recruited and subsequently transformed (Wang et al., 2017; Balkwill, Capasso & Hagemann, 2012). The existence of the TME is prompted and perpetuated by conditions that exist and arise, such as hypoxia and inflammation (Jiang et al., 2020).

The cellular components of the TME include a variety of cell types, including immune cells such as macrophages and lymphocytes, as well as fibroblasts, adipocytes, bone-marrow derived cells, dendritic cells, stem cells and vascular cells, such as endothelial cells and pericytes (Egeblad, Nakasone & Werb,

2010; Egeblad et al., 2008). Similarly, the range of non-cellular components of the TME are vast and include the ECM and its structural and non-structural components; soluble factors, such as growth factors, cytokines, enzymes and angiogenic factors; and mediators of paracrine signals, such as regulatory ribonucleic acids (RNAs) and metabolites (Patel et al., 2018).

A healthy stromal microenvironment is key to normal tissue functioning and wound healing as previously mentioned. Physiological wound healing is tightly controlled and functional. In tumours, which have been described as chronic wounds that do not heal, stromal cells are co-opted to provide a pathological wound healing response that is unregulated and classified as hyperactivated (Huet et al., 2019; Dvorak, 2015). Therefore, once stromal activity and signalling are altered, it contributes to the characteristic dysregulation of various signalling pathways and cellular responses responsible for cancer progression (Hanahan & Weinberg, 2011; Wiseman & Werb, 2002). The presence of the TME is thus key to cancer progression – impacting tumour initiation, growth, invasion, metastasis and treatment response (Zhang, Nie & Chakrabarty, 2010).

As these cells are transformed, they acquire a tumour or a tumour-associated phenotype. Examples are macrophages and fibroblasts that are transformed to tumour-associated macrophages and cancer-associated fibroblasts (CAFs), respectively (Shiga et al., 2015; van Overmeire et al., 2014). Stromal endothelial cells have similarly been shown to be key to cancer progression – being transformed to tumour endothelial cells (TECs).

1.4.1 Tumour Endothelial Cells (TECs)

Endothelial cells are altered in all pathologies, including cancer and within the TME, they are transformed into TECs (Carson-Walter et al., 2001; St. Croix et al., 2000). Cancer cells signal and stimulate endothelial cells by producing growth factors and cytokines. Yet, their relationship is bi-directional, where endothelial cells influence cancer cells, and their crosstalk influences the release of soluble factors, including angiogenic growth factors (Buchanan et al., 2012).

TECs, which line tumour blood vessels, function as defective, hyperactivated endothelial cells with structural and functional abnormalities. They may result from direct or indirect crosstalk between healthy endothelial cells and cancer cells, as well as conditions such as hypoxia present within the TME, and they are also influenced by epigenetic regulation (Ciesielski et al., 2020; Lopes-Bastos, Jiang & Cai, 2016; Hashizume et al., 2000). While stromal endothelial cells are an important source, additional sources of TECs include circulating (mature) endothelial cells, cancer cells and vascular progenitor cells

(**Figure 1.3**), all contributing to the abnormality of TECs and the heterogeneity of the TEC population (Hida et al., 2018).

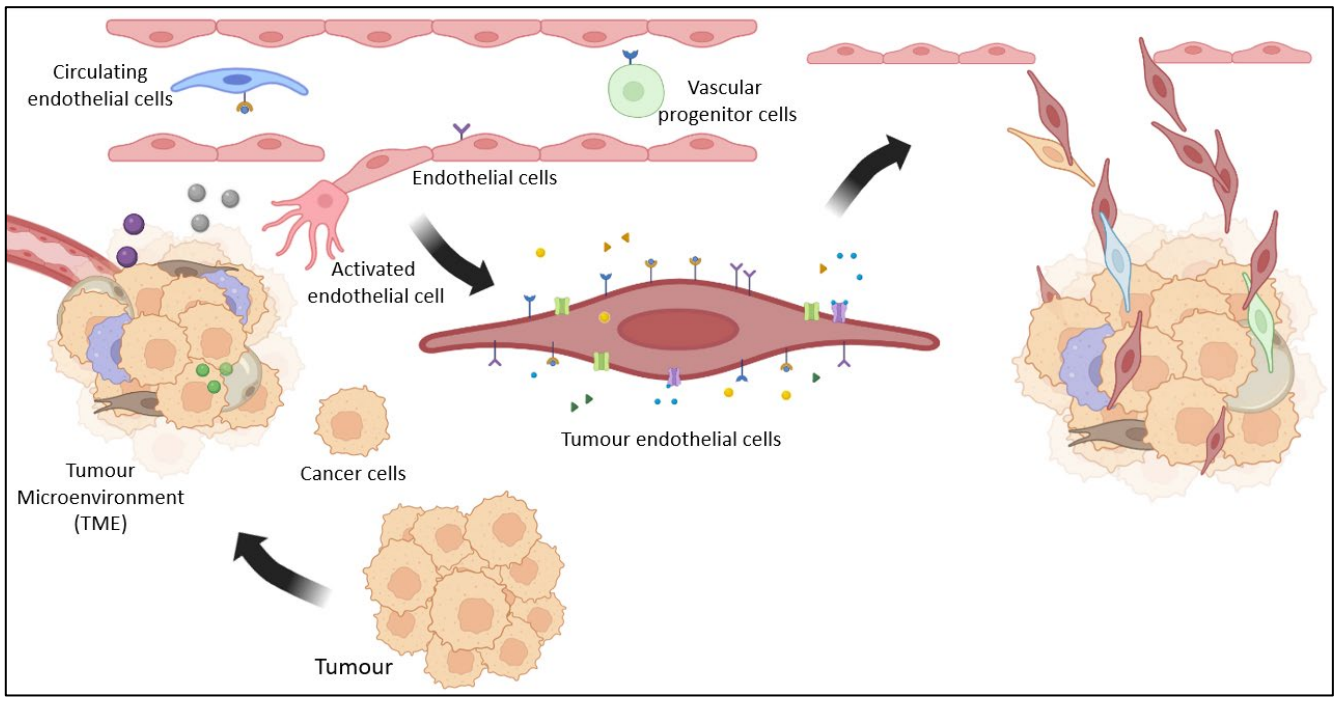

Figure 1.3: Tumour endothelial cells are a consequence of the tumour microenvironment. Sources of tumour endothelial cells (TECs) include surrounding endothelium, circulating endothelial cells, vascular progenitor cells and cancer cells themselves. TECs are defective hyperactive endothelial cells that line tumour blood vessels or present within the tumour microenvironment (TME). They are a consequence of direct or indirect crosstalk between 'normal' endothelial cells (non-transformed) and cancer cells, conditions present within the TME, and epigenetic regulation. They have irregular morphology and form defective blood vessels; they are highly proliferative and increasingly responsive to growth factors; they are pro-angiogenic and anti-apoptotic, with a short life span; and they are aneuploid and possess chromosomal abnormalities (Created in Biorender.com).

Various characteristic features of TECs have been recorded. The rate of endothelial turnover in malignant tissue may be 20-2000 times faster than the rate in normal cells, which is generally low and declines with age (Hobson & Denekamp, 1984; Schwartz & Benditt, 1977). TECs are aneuploid, while normal endothelia are diploid, and have abnormal chromosomes, resulting in chromosomal instability (Hida et al., 2018). These cells do not undergo senescence as is characteristic of normal endothelial cells, they are more resistant to apoptosis and are more sensitive to growth factors, with a pro-angiogenic phenotype (Hida et al., 2018; Bussolati et al., 2003). Furthermore, morphological

abnormalities observed in TECs are irregular sizes and shapes, as well as ruffled margins and superficial cytoplasmic projections that are long and fragile, extending across the blood vessel's lumen (Mcdonald & Choyke, 2003).

The contribution of TECs to cancer progression is extensive, including sustained tumour angiogenesis, immune response, drug resistance, tumour growth, and invasion and metastasis (Bussolati et al., 2003; Dudley et al., 2008; Fessler et al., 2015; Ingthorsson et al., 2010; Maishi et al., 2016; Nagl et al., 2020; Sigurdsson et al., 2011). There are a variety of molecules that serve as endothelial and TEC markers that contribute to the sustained angiogenesis, drug resistance, immune response and stemness associated with these cells.

1.4.1.1. Tumour endothelial cell (TEC) markers

Along with the phenotypic changes, genetic changes are induced by crosstalk with pro-tumourigenic cells and the local TME. This leads to the upregulation of molecular markers that are not normally expressed or expressed at low levels in normal and quiescent endothelial cells (Hida, Hida & Shindoh, 2008; Hotchkiss et al., 2005; St. Croix et al., 2000). Yet, little consensus exists pertaining to markers specific to TECs or which markers are best in identifying TECs. TEC markers consist mostly of molecules, including genes, receptors and proteins that are expressed or overexpressed in TECs compared to normal endothelial cells. The presence of these markers is largely responsible for the abnormal features of TECs and may potentially serve as therapeutic targets (Yamada et al., 2015; St. Croix et al., 2000). A selection of markers reported to be overexpressed in TECs has been reviewed in **Table 1.1.1**.

Table 1.1: A review of TEC markers.

Basic description and function	Processes in cancer	Endothelial cell functions	TEC functions
Vascular endothelial growth factor receptor 2 (VEGFR2)			
A Tyrosine kinase receptor. The predominant VEGFR and receptor for VEGF-A (VEGF), but also binds VEGF-C & VEGF-D. Key to the development and maintenance of the circulatory and lymph vascular systems. A main driver of vasculogenesis, angiogenesis and lymph-angiogenesis. (Karaman et al., 2022; Wang et al., 2020)	Involved in tumour growth, tumour angiogenesis and endothelial transdifferentiation of tumour cells, invasion and metastasis and recurrence. Known to promote tumourigenicity. (Lian et al., 2019; Lu et al., 2019; Liu et al., 2017)	Important for cell communication, proliferation, differentiation, migration, and vascular permeability. (Karaman, Leppänen & Alitalo, 2018; Shibuya, 2013; Stuttfeld & Ballmer-Hofer, 2009; Holmes et al., 2007)	Enhanced expression confers hypersensitivity to VEGF signalling. No different roles specified compared to normal endothelial cells. Key to proliferation and cell survival. (Miettinen et al., 2012; Matsuda et al., 2010; Smith et al., 2010; Amin et al., 2008; Bussolati et al., 2003)
Multidrug Resistance 1 (MDR1)			
A gene encoding P-glycoprotein, an energy-dependent integral membrane transporter. It functions as a protective efflux pump that removes metabolites, substrates and harmful molecules, including drugs, present within the cell or cell membrane. (Chen, Sun, et al., 2020; Rockwell, 2004; Brinkmann & Eichelbaum, 2001; Hoffmeyer et al., 2000; Ueda et al., 1986)	Drug resistance and cross-resistance to multiple unrelated drugs, despite only being exposed to one regime. (Rockwell, 2004; Ling & Thompson, 1974)	Most prominent in endothelial cells specialized for barrier function. It has an embryonic and stem component. Rare in quiescent endothelial cells and vascular progenitor cells. (Krawczenko et al., 2017; Konieczna et al., 2011; Sun et al., 2006; Melaine et al., 2002)	Drug resistance activated downstream of angiogenic signalling and upon exposure to cytoxic drugs. (Akiyama et al., 2012)

Biglycan			
A ubiquitously expressed proteoglycan. It is a structural component of the ECM that, when available in its soluble form – whether through matrix degradation or cell secretion, acts as a signalling molecule in a variety of systems. (Nastase, Young & Schaefer, 2012; Schaefer & Schaefer, 2010; Babelova et al., 2009)	Tumourigenic role is dependent on its cellular origin. Pro-tumourigenic roles include tumour growth, invasion and metastasis, as well as tumour angiogenesis. (Aggelidakis et al., 2018; Subbarayan et al., 2018; Xing et al., 2015; Hu et al., 2014).	Almost undetectable under physiological conditions. Well-established role in angiogenesis, contributing to cell migration, tube formation, tubulogenesis - lumen formation, cell proliferation and VEGF expression. Involved in the regulation of vessel formation. (Hu et al., 2016; Berendsen et al., 2014; Obika et al., 2013; Calabrese et al., 2011)	Key to tube formation and VEGF chemotaxis and influences cell morphology. Implicated in TEC-driven metastasis. (Maishi et al., 2016; Yamamoto et al., 2012)
Lysyl Oxidase (LOX)			
A copper-dependent amine oxidase. Through its cross-linking of ECM proteins, collagen and elastin, its main function is as an ECM-modifying enzyme. (di Stefano et al., 2016; Smith-Mungo & Kagan, 1998)	Contributes to tumour growth, regrowth and metastasis but has been observed to function as a tumour suppressor. (Huang et al., 2018; Rachman-Tzemah et al., 2017; di Stefano et al., 2016; Cox et al., 2015; Baker et al., 2011, 2012; Laczko et al., 2007; Payne, Hendrix & Kirschmann, 2007; Payne et al., 2005).	Key to endothelial barrier integrity, the maturation and stability of the ECM. Influences cell proliferation. Tightly regulated expression to maintain vascular homeostasis. (Adamopoulos et al., 2016; Rodríguez et al., 2008)	Pro-tumourigenic, labelled as a pro-angiogenic activator in TECs. Enhances cell motility, migration, tube formation and alters cell morphology. Contributes to metastasis, dissemination of circulating tumour cells (CTCs) and promotes VEGF secretion. (Baker et al., 2013; Osawa et al., 2013)

Tumour endothelial marker 7 (TEM7)			
A cell surface protein, involved in cell attachment, adhesion and migration. Functions are attributed to its structural domains and binding partners, nidogen and cortactin. (Schnoor, Stradal & Rottner, 2018; Konwerska, Janik & Malinska, 2011; Lee et al., 2005; Nanda, Buckhaults, et al., 2004; Chung et al., 1993)	Linked to tumour differentiation, inflammation, invasion, metastasis and CTCs, and is a prognostic biomarker of poor survival. (Fuchs et al., 2007; Geng et al., 2021; Pietrzyk & Wdowiak, 2019; Zhang et al., 2015)	Classified as a vascular protein and associated with angiogenic states. Involved in capillary morphogenesis. (Bagley et al., 2011; Wang, Sheibani & Watson, 2005)	Most abundantly expressed TEM in the tumour vasculature. Thought to play a key role in tumour angiogenesis, yet its exact functions are unknown. It is also associated with a vascular progenitor phenotype and circulating (mature) endothelial cells. (Mehran et al., 2014; Bagley et al., 2011; Beaty et al., 2007; Nanda & St. Croix, 2004; Nanda, Buckhaults, et al., 2004)
Tumour endothelial marker 8 (TEM8)			
An integrin-like cell surface receptor. Known as Anthrax toxin receptor 1 (ANTXR1) – binds the protective antigen sub-unit of the anthrax toxin protein. Anthrax is a rare but lethal infectious disease caused by the *Bacillus thracis* bacterium. Not fully understood but appears to regulate the cytoskeleton by interacting with collagens. (Pietrzyk, 2016; Yang et al., 2011; Fu et al., 2010; van der Goot & Young, 2009; Werner, Kowalczyk & Faundez, 2006)	Important for cancer progression, being key to tumour growth, tumour angiogenesis and vascular mimicry, metastasis, and survival. (Xu et al., 2021; Høye et al., 2018; Cao et al., 2016; Opoku-Darko et al., 2011; Nanda, Carson-Walter, et al., 2004; Rmali et al., 2004)	TEM8 is involved in embryonic angiogenesis and is not associated with physiological 'adult' angiogenesis. It contributes to endothelial cell differentiation, tube formation and cell motility. It's a crucial component of the migratory process, and influences cell-matrix interactions. (Cao et al., 2016; Besschetnova et al., 2015; Hotchkiss et al., 2005; Carson-Walter et al., 2001)	No exact functions defined yet. Associated with vascular progenitor cells as biomarkers for cancer. (Mehran et al., 2014)

Chemokine CXC receptor 7 (CXCR7)			
A β-arrestin coupled chemokine receptor encoded by the *RDC1* gene, also known as Atypical Chemokine receptor 3. Functions as a scavenger receptor for Chemokine CXC ligand 12 (CXCL12) and CXCL11/interferon-inducible T cell α chemoattractant (CXCL11) and possesses the ability to dimerize with CXCR4. (Shimizu et al., 2011; Naumann et al., 2010; Rajagopal et al., 2010; Burns et al., 2006; Balabanian et al., 2005).	Important for cancer progression, playing a role in tumour growth, tumour angiogenesis, vascular dysfunction, and metastasis (Li et al., 2020; Liu et al., 2020; Qian et al., 2018; Totonchy et al., 2014; Miao et al., 2007).	A crucial angiogenic inducer, influencing proliferation pathways, cell polarization, migration, and tube formation. Vasculogenic effects are seen in vascular progenitor cells, influencing cell adhesion and migration. (Qian et al., 2018; Zhang et al., 2017; Yamada et al., 2015; Dai et al., 2011; Liu et al., 2010; Costello et al., 2008; Burns et al., 2006)	Prompts cell proliferation and confers resistance to serum starvation. Enhances cell motility, migration and adhesion, also alters cell morphology. Confers loss of contact inhibition and promotes tube formation, as well as the formation of defective vessel barriers (Yamada et al., 2015; Totonchy et al., 2013, 2014; Burns et al., 2006).

1.4.2. Tumour angiogenesis

Tumour angiogenesis, in which TECs play a central role, is a well-established hallmark of cancer, significantly impacting tumour progression and treatment response (Hanahan, 2022; Frentzas, Lum & Chen, 2020; Jiang et al., 2020; Lugano, Ramachandran & Dimberg, 2020; Klein, 2018; Zuazo-Gaztelu & Casanovas, 2018; He et al., 2014; Hanahan & Weinberg, 2011; Craft & Harris, 1994). It is the consequence of a tumour switching from an avascular phase to a vascular phase. This transition is initiated by an “angiogenic switch” – when the tightly regulated balance of anti-angiogenic to pro-angiogenic factors is overpowered by the plethora of pro-angiogenic factors (Bergers & Benjamin, 2003; Ribatti, Vacca & Dammacco, 1999). Cancer cells possess the ability to ‘elicit continued growth of new capillary endothelium from its host’ (Algire et al., 1945). Tumour angiogenesis can, therefore, be defined as chronically activated angiogenesis within the TME.

Angiogenesis in a tumour is triggered when the size of the tumour exceeds 1 to 4 mm in diameter – leading to an insufficient supply of oxygen and nutrients as the tumour outgrows the network of existing blood vessels (Folkman, 1990). Tumour angiogenesis is essential for tumour growth, while the absence of angiogenesis may act as a rate-limiting step. It is also required to permit metastasis (Craft & Harris, 1994; Folkman, 1990). The main drivers of tumour angiogenesis are elevated and continuous stimulation of endothelial migration, proliferation, and blood vessel remodelling (Fox et al, 1993). Tumour angiogenesis can be classified into angiogenic responses and non-angiogenic responses. Angiogenic responses denote the involvement of endothelial cells and non-angiogenic responses denote the involvement of non-endothelial cells that acquire an endothelial phenotype or mimic their behaviour, as depicted in **Figure 1.4**.

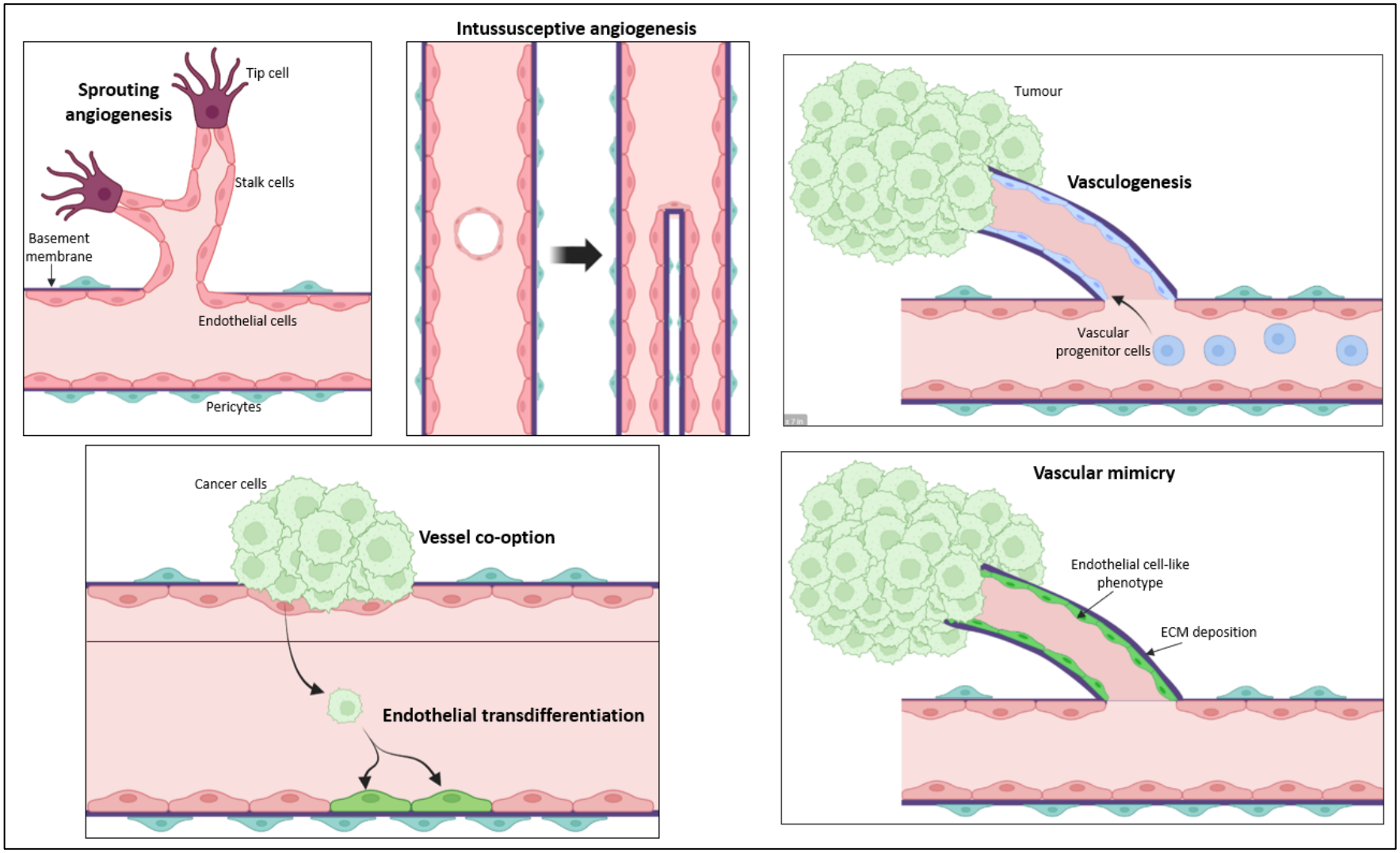

Figure 1.4: Blood vessel formation strategies implicated in tumour angiogenesis. Tumour angiogenesis is comprised of angiogenic strategies: sprouting angiogenesis, vasculogenesis and intussusceptive angiogenesis; and non-angiogenic strategies: vessel co-option, endothelial transdifferentiation, and vascular mimicry (Adapted from Eelen et al., 2020) (Created in Biorender.com). Abbreviations: ECM, Extracellular matrix

1.4.2.1 Angiogenic vascularization

Tumour angiogenesis differs from physiological angiogenesis for several reasons. Tumour angiogenesis is comprised of SA, IA and vasculogenesis (Karthik et al., 2018; Betz et al., 2016). SA precedes IA, which remodels the existing vessels (Karthik et al., 2018). Neovascularization by SA and IA are regulated by hypoxia, soluble factors and TME interactions.

The chronic state of hypoxia in tumours is mediated by HIF-1α and HIF-2α. The hypoxic state is driven by the rapid proliferative rate of cancer cells that leads to the absence of oxygen, which persists even in highly vascularized tissues because of dysfunctional blood vessels (Krock, Skuli & Simon, 2011; Nejad et al., 2021; Holmquist-Mengelbier et al., 2006; Korbecki et al., 2021). It is further perpetuated by conditions such the generation of ROS and mutations that lead to the overexpression of HIF-α subunits. HIF-α degradation may also be inhibited through various mechanisms, including the inhibition of propyl hydroxylase enzyme activity responsible for the degradation of HIF-α subunits (Ravi et al., 2000; Kikuchi et al., 2009; Selak et al., 2005).

The plethora of oncogenes that increase the expression of VEGF, additional angiogenic inducers which are dependent or independent of VEGF, gene overexpression in cancer cells. as well as altered stromal cells, all significantly impact tumour angiogenesis. These changes are influenced by stromal interactions, hypoxia and mutations that impact regulatory mechanisms (Nejad et al., 2021; Kerbel & Folkman, 2002; Buchanan *et al*., 2011). A broader range of pathways and angiogenic factors are active in the TME. Examples of key pathways that contribute to tumour angiogenesis include VEGF-A and its receptors; Delta-like and Jagged ligands and their Notch receptors; and angiopoietins and their Tie receptors, Tie1 & Tie2 (Ziyad & Iruela-Arispe, 2011). Additional growth factors implicated include fibroblast growth factors (FGF), TGF- β1 and PDGFs (Buchanan *et al*., 2011).

Vasculogenesis denotes the involvement of vascular progenitor cells in blood vessel formation. The most potent activator of the vasculogenic pathway is stromal cell-derived factor 1, known as CXCL12. CXCL12 is a chemokine ligand that binds CXCR4 and CXCR7 to promote the recruitment of vascular progenitor cells and other bone-marrow derived cells with vascular modulatory functions, such as myelomonocytic cells (Song et al., 2017; Brown, 2014; Kioi et al., 2010; Kozin et al., 2010). Fibroblasts serve as the major source of CXCL12 through: (1) the transformation to CAFs; and (2) downstream HIF activity during a chronic state of hypoxia (Orimo et al., 2005; Hitchon et al., 2002).

An additional contributing factor is the recruitment and adaptation of additional cells that participate in blood vessel formation (Witsch, Sela & Yarden, 2010; Ferrara & Kerbel, 2005). Pericytes and pericyte like cells are also altered in the TME, e.g., altered signalling due to the chronic presence of VEGF can influence the degree of pericyte coverage, where low degrees of pericyte coverage

contributes to vessel destabilization and inhibition of vessel maturation (Ribeiro & Okamoto, 2015; Greenberg et al., 2008). Furthermore, the angiogenic process is tumour-specific, with varying intensities and mural cell involvement (Eberhardt et al., 2000).

1.4.2.2 Non-angiogenic responses

Tumours can also employ non-angiogenic strategies to form their vasculature, namely, vessel co-option, vascular mimicry and endothelial transdifferentiation. These strategies allow tumours to progress and metastasize without, or in addition to, the hallmark process of angiogenesis initiated to recruit new blood vessels (Leenders et al., 2004; Qian et al., 2016).

In vessel co-option, tumours take over the host organ's vasculature present in the non-malignant tissue (Kuczynski et al., 2019; Donnem et al., 2013). The pre-existing vasculature may be hijacked by cancer cells migrating along the abluminal surface of the vessels or invading the tissue space between the vessels (Kuczynski et al., 2019). It is subsequently accompanied by vessel remodeling, during which the non-malignant vasculature is transformed into tumour vasculature (Kim et al., 2002). Incorporation of the hijacked blood vessels provides both a blood supply and a metastatic route. It is thought to occur in tissues that are highly vascularized, such as the brain; at the vascularized edges of growing tumours; or in the initial stages of tumour formation (Donnem et al., 2013; Leenders et al., 2004; Kim et al., 2002).

In vascular mimicry, cancer cells mimic endothelial cells by forming hollow tubes that are embedded within the ECM. These tubes are perfused and fully functional as blood vessels in the absence of endothelial components and are thus classified as cancer cell-derived vasculature (Maniotis et al., 1999; Qian et al., 2016). This ability is associated with an aggressive phenotype and the accompanying high degree of plasticity is further exacerbated by hypoxia (Mihic-Probst et al., 2012; Seftor et al., 2012; Maniotis et al., 1999). These cells possess a genotype indicative of a pluripotent embryonic state and exhibit upregulated expression of a variety of genes, including genes that are associated with endothelial cells and their related processes, such as blood vessel formation and coagulation inhibition (Seftor et al., 2012; Bittner et al., 2000).

In endothelial transdifferentiation, cancer stem-like cells differentiate into functional endothelial cells, then referred to as tumour-derived endothelial cells, that participate in tumour angiogenesis. This change is associated with an aggressive phenotype and cancer stemness (Chen & Wu, 2016; Seftor et al., 2012; Soda et al., 2011; Zhao et al., 2018). The accepted mechanism of action is that hypoxia triggers the differentiation of cancer cells into cancer stem cells, which subsequently differentiate into endothelial cells (Chen et al., 2020; Zhang et al., 2013). The occurrence of these mechanisms

significantly contributes to tumour progression and are key adaptations to conditions within the TME, as well as during anti-angiogenic treatment, thereby further complicating cancer therapy (He et al., 2014).

The addition of non-angiogenic responses complicates the description of the phenomenon of tumour angiogenesis. It increases the scope of cell types, pathways and soluble factors involved, as well as the type of tumour, which all add complexity to this scenario. The blood vessels which are generated, are however, distinct with general identifiable features when compared to normal blood vessels.

1.4.2.3 Tumour blood vessels

Despite being a functional physiological process, in cancer the plethora of angiogenic signals do not cease and surpass the regulatory mechanisms in place. Therefore, as opposed to the normal, optimally functioning blood vessels with a hierarchal branching pattern of arteries, veins and capillaries, the blood vessels generated within the tumour are disorganized and abnormal. They are hyperpermeable, which increases the interstitial fluid pressure in the tumour; they have reduced blood flow and have dysregulated vessel diameters (Senthebane et al., 2017; Uldry et al., 2017; Hida, Hida & Shindoh, 2008).

In normal blood vessels the endothelial cells exist in a monolayer and form tight junctions between the cells. In tumour blood vessels, the monolayer and endothelial interconnections are defective with excessive branching and abnormal sprouting. This also impairs normal barrier functioning and results in the formation of branches that extend across the vessel's lumen (Hida, Hida & Shindoh, 2008; Mcdonald & Choyke, 2003). This hyperactivated process is associated with a change in endothelial shape, which consequently results in the formation of intercellular gaps that contribute to vessel leakiness, allowing blood, fluid and fibrin to leak into the surrounding tissue (Hashizume et al., 2000).

Along with small openings between neighbouring TECs, larger intercellular openings and holes may exist within the vessel wall, contributing to tumour haemorrhage (Hashizume et al., 2000; Mcdonald & Choyke, 2003). The large openings are attributed to the abnormally high turnover rate, which may prevent the proper formation of basement membranes and intercellular junctions. Vessel maturity is further impacted by the transformation of mural cells, like pericytes (Morikawa et al., 2002). Additionally, cancer cells compress the immature vessels, which results in an uneven diameter, and the high interstitial pressures that exist in tumours may cause the vessel to collapse and prevent blood flow (Hida, Hida & Shindoh, 2008; Mcdonald & Baluk, 2002).

While newly formed blood vessels recede once tissues are supplied, blood vessels stay at pathologically high numbers as angiogenesis is continually triggered and abnormal, ineffective sprouts generated (Dudley, 2012; Algire et al., 1945). These abnormalities are crucial contributors to the abnormal TME that allow cancer cells to survive, thrive and propagate. Blood vessels also play a key role in the success of cancer therapies, where abnormal vessels prevent drugs from reaching their targets. The abnormal vessels, as well as endothelial cells and angiogenesis have thus become a target in treatment regimens with anti-angiogenic therapy.

1.4.2.4 Anti-angiogenic therapy

The broad basis of anti-angiogenic therapy is rooted in the importance of angiogenesis and endothelial cells to tumour progression, whereas anti-VEGF therapy is centred around the best characterized role of the VEGF family in the initiation and mediation of angiogenic and tumour angiogenic processes (Grothey & Ellis, 2008; Ferrara & Kerbel, 2005). Anti-angiogenic agents can range from agents that inhibit several steps in the angiogenic process to ones that specifically target certain molecules, as seen with anti-VEGF regimes (Grothey & Ellis, 2008; Sato, 2003).

The main target of anti-angiogenic therapy is the event of angiogenesis and inhibiting or delaying its processes such as proliferation. Success has been seen in the use thereof, especially in highly vascularized tumours and in combination with other cancer treatment regimes (Lu-Emerson et al., 2015; Lacouture, Lenihan & Quaggin, 2009). There are, however, several limitations. The angiogenic process not only involves endothelial cells, but mural cells and other microenvironmental cells, which can significantly influence the response to anti-angiogenic agents (Helfrich & Schadendorf, 2011). Vasculogenesis also serves as a ‘back-up’ angiogenic response and therefore contributes to tumour recurrence (Kioi et al., 2010).

Non-angiogenic vasculature responses and adaptations also contribute to anti-angiogenic drug resistance (Donnem et al., 2013; Soda et al., 2011). Anti-angiogenic therapy, such as anti-VEGF agents, have the potential to increase tumour aggressiveness, which complicates further treatment (Lupo et al., 2017; Pàez-Ribes et al., 2009). Additionally, anti-angiogenics may promote endothelial transdifferentiation (Soda et al., 2011). Furthermore, the impact of anti-angiogenics is limited on overall patient survival and often requires selection of suitable candidates, not being suitable for general use (Zirlik & Duyster, 2018; Lu-Emerson et al., 2015).

There are numerous adverse effects associated with anti-angiogenic therapies and especially the use of agents targeting the VEGF and PDGF receptor families (Lupo et al., 2017; Burger et al., 2011; Lacouture, Lenihan & Quaggin, 2009). Cardiovascular complications include hypertension, thrombosis, bleeding and even heart failure. Other serious adverse effects include complications

related to wound healing, gastrointestinal perforations, hypothyroidism, skin reactions and renal complications such as proteinuria. Less life-threatening effects include dermatological reactions such as hand-foot skin reaction, skin discoloration and dryness, and alopecia (Elice & Rodeghiero, 2012; Burger et al., 2011; Chen & Cleck, 2009; Lacouture, Lenihan & Quaggin, 2009).

Two shifts have been made in the paradigm of angiogenic therapy. The one involves a shift to pro-angiogenic approaches, based on the ability of optimally functioning blood vessels to deliver drugs and provide their maximal effect (Lupo et al., 2017). The other focuses on specifically targeting TECs with antiangiogenic therapy. The ability to isolate TECs has provided immense advances in understanding tumour biology, the tumour vasculature, the role of TECs and key signalling pathways, as previously discussed (He et al., 2014). Efforts made in this approach have been targeting markers observed to be specific to the TEC phenotype and central to the tumour angiogenic process, with the use of antibodies and have proven to have significant effects in *in vitro* and *in vivo* preclinical data (Fonsatti et al., 2010). Anti-angiogenic therapy has relevance in various diseases and the targeting of TECs provides avenues to optimize efficacy, mitigate treatment resistance and eradicate adverse effects (He et al., 2014).

1.5 Problem statement

Interactions between cancer cells and their microenvironment contribute to cancer progression and treatment efficacy. Establishing a TME and altering cell phenotypes to tumour or tumour-associated phenotypes are, therefore, key determinants in the disease pathology (Son et al., 2017). Research has shown that endothelial cells contribute to cancer progression through the transformation to a TEC phenotype, leading to dysregulated tumour angiogenesis. Targeting tumour blood vessels in cancer therapy has proven to be an important strategy yet presents major limitations with the frequent occurrence of resistance, enhanced tumourigenicity and side effects. Focus has thus shifted to target TECs directly and thereby stabilizing tumour blood vessels. The approach to targeting TECs is through a wide array of characteristics that have been attributed to the TEC phenotype and not a normal endothelial cell phenotype. Various components of the microenvironment in breast cancer have been investigated, yet research pertaining to how breast cancer affects the endothelium and angiogenesis is limited.

1.5.1 Research questions

The problem presented leads to the following research questions:

1. Do breast cancer cells induce a TEC phenotype in endothelial cells?

2. How does breast cancer impact the angiogenic response?
3. How does the response in endothelial cells induced by breast cancer cells differ to that induced by non-malignant breast cells?

1.5.2 Hypothesis

We hypothesize that breast cancer cells induce a TEC phenotype and hyperactivate the angiogenic response compared to non-malignant breast epithelial cells.

1.5.3 Aims

The aims of the study are to:

1. Evaluate the TEC phenotype in an *in vitro* breast model.
2. Assess the paracrine influence of breast cancer on angiogenic processes.

1.5.4 Objectives

The aims of the study will be determined by utilizing the following objectives:

1. To harvest conditioned medium (CM) from non-malignant and malignant breast cell lines.
2. Assess cell viability of endothelial cells treated with breast cell CM using a Water-soluble tetrazolium salt-1 (WST-1) assay.
3. Assess cell morphology of endothelial cells treated with breast cell CM using phase contrast imaging.
4. Assess TEC gene expression following treatment with breast cell CM using reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR).
5. Assess TEC protein expression following treatment with breast cell CM using western blots.
6. Assess cell migration of endothelial cells treated with breast cell CM using a scratch assay.
7. Assess tube formation of endothelial cells treated with breast cell CM using a tube formation assay.

Chapter 2: Methods and materials

2.1 Cell culture

To assess the influence of breast cancer cells on endothelial cells, the biological model employed was an *in vitro* cell culture model using MCF-7 and MDA-MB-231 breast cancer cells, MCF-12A non-malignant breast cells and human umbilical vein endothelial cells (HUVECs) (**Table 2.1**). MCF-7 cells are luminal-A breast cancer cells that are estrogen and progesterone receptor-positive. They are non-invasive epithelial breast cancer cells that are less aggressive and possess a low metastatic potential (Comşa, Cîmpean & Raica, 2015). MDA-MB-231 cells are highly invasive TNBC cells with gene expression that is predominantly mesenchymal (Blick *et al*., 2008). MCF-12A cells are non-malignant breast epithelial cells (Sweeney et al, 2018). HUVECs are human umbilical cord endothelial vein cells that provide insight into the physiological and pathophysiological responses of the human vascular endothelium. As such, HUVECs have proven to be useful in cancer-related studies (Medina-Leyte et al., 2020).

Table 2.1: Cell line descriptions.

Cell line	Description	Phenotype
MCF-12A	Non-malignant epithelial cells.	Epithelial
MCF-7A	Luminal A breast cancer cells, which are ER, PR and HER-2 positive.	Epithelial Low proliferative rate Non-metastatic
MDA-MB-231	Basal-like triple negative breast cancer cells, lacking ER, PR and HER-2.	Mesenchymal Metastatic Highly proliferative
Human umbilical vein endothelial cells (HUVECs)	Derived from the umbilical vein.	Endothelial

Complete culture medium for MCF-7 and MDA-MB-231 cells was prepared using Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, ThermoFisher Scientific®, Cat # 41965-062) supplemented with 1% Penicillin-Streptomycin (PenStrep) (Gibco™, ThermoFisher Scientific®, Cat # 15140-122) and 10% Fetal Bovine Serum (FBS) (Capricorn Scientific®, ThermoFisher Scientific®, Cat # FBS-G1-12A). DMEM will be used to refer to DMEM supplemented with PenStrep, and complete DMEM will be used to refer to DMEM supplemented with PenStrep and FBS.

Complete culture medium for MCF-12A cells was prepared using DMEM and Ham's F-12 Nutrient Mix (Gibco™, ThermoFisher Scientific® Cat # 11765054) supplemented with 1% PenStrep, 500 ng/mL hydrocortisone (LKT Laboratories, Cat # H9611), 20 ng/mL EGF (R&D Systems, Cat # 236-GMP), 10 µg/mL insulin (Lilly, Humulin 30/70, Cat # 783811004), 100 ng/mL cholera toxin (Sigma-Aldrich, Cat # C8052) and 10% FBS. Complete culture medium for HUVECs, donated by Dr Amanda Genis (Stellenbosch University), was prepared using MCDB-131 culture medium (Gibco™, Cat # 10372-019) supplemented with 1% PenStrep, Glutamax (Gibco™, Cat # 35050038), 1 µg/mL hydrocortisone, 10 ng/mL EGF, 5 ng/mL basic FGF (bFGF) (Gibco™, ThermoFisher Scientific®, Cat # 13256-29) and 5% FBS, according to a recipe established by Prof Niel Davies (University of Cape Town). HUVECs were used between passages 4-5 for experimental purposes.

Cells were maintained at 37°C in a 5% CO_2 incubator. Cells were cultured in T25 or T75 flasks and sub-cultured at 70-80% confluency. For all the breast cells, once confluent, cells were trypsinized (0.25% Trypsin-EDTA (Gibco®, ThermoFisher and Scientific®, # 25200072)) until 90% of the cells were detached. Culture medium was added to neutralize the trypsin and the cell suspension was centrifuged for 3 minutes at 1500 rpm. For HUVECs, once confluent, the cells were rinsed with HEPES Buffered Saline Solution (Lonza, Cat # CC-5022) and trypsinized until 90% of the cells were detached. Trypsin neutralizer (ThermoFisher and Scientific®, Cat # R002100) was added and the cell suspension was centrifuged for 5 minutes at 200 g. The supernatant was discarded, and cells were seeded in the respective culture flasks or plates. The cell densities and time to confluence used for the different cell lines and assays are listed in **Table 2.2**. Cells were seeded at higher densities for the scratch and tube formation assays. The scratch assay requires cells to be at confluence (100%) when the experiment is started and densities for tube formations were optimized due to tubes not forming at lower densities.

Table 2.2: Densities and adherence times used per cell line for the respective assays.

Cell line	Cell densities per flask/plate		Time to confluence	Assays
MCF-12A	3 000 000	T175	24 hours	CM harvest
MDA-MB-231	5 000 000	T175	24 hours	
MCF-7	4 500 000	T175	48 hours	
HUVECS	12 500	48-well	24 hours	WST-1 assay
	180 000 & 300 000	24-well		Scratch & tube formation assay
	67 500	12-well		RT-qPCR
	180 000	6-well		Western blots

2.2 Conditioned medium (CM) generation and treatment

For each treatment group, cells were seeded in T75 or T175 flasks, allowed to grow to confluency and treated with DMEM (MCF-7 and MDA-MB-231) & DMEM/Hams (MCF-12A). Cells were treated with complete culture medium or culture medium starved of all growth supplements. After the 24-hour treatment period, the CM was collected, and centrifuged at 4°C for 10 minutes at 5000 rpm. The supernatant was subsequently transferred to a new tube and filtered using a syringe and 0.22 µM filter. Samples were snap frozen and stored at -80°C. Once all the CM was harvested, it was thawed and pooled, aliquoted for each respective assay, snap frozen, and stored at -80°C until further analysis.

Cells (HUVECs) were seeded in cell culture plates, incubated until 70-90% confluency and treated with pre-warmed (37°C) cell culture medium (Control) and CM (MCF-12A, MCF-7 or MDA-MB-231) for 24 hours and used for subsequent analysis, as depicted in **Figure 2.1**. All culturing experiments were performed in duplicate or triplicate, and each experiment was repeated for a minimum of two or three experimental repeats for qualitative analysis and quantitative analysis, respectively.

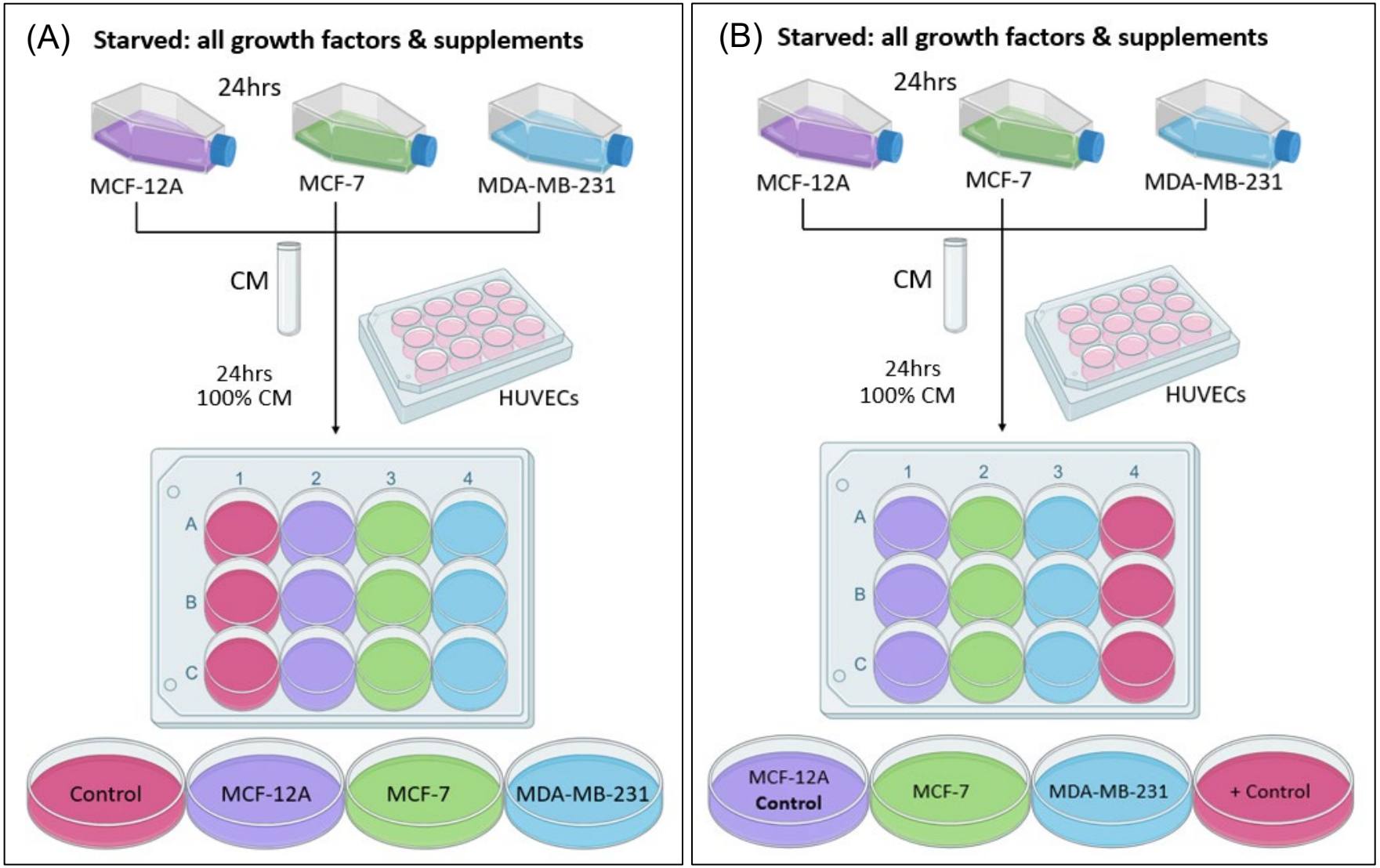

Figure 2.1: Treatment approach used to address (A) aim 1 and (B) aim 2. Abbreviations: CM, Conditioned medium; HUVECs, Human umbilical vein endothelial cells (Created in Biorender.com).

2.3 Cell viability assays

2.3.1 Water-soluble tetrazolium salt-1 (WST-1) assay

The Water-soluble Tetrazolium Salt-1 (WST-1) assay measures mitochondrial reductive capacity, which serves as an indirect measurement of cell viability and cell proliferation. For a WST-1 assay, following the previously described treatment protocol (**Figure 2.1-A**), cells were incubated with the WST-1 reagent. WST-1 (BiocomBiotech, Cat # AB155902) was thawed and warmed to 37°C in preparation for the experiment. Each experimental well was treated with 20 µL of WST-1 (1:10) 90 minutes prior to end of the treatment period. After incubation, plates were briefly shaken on a shaker, and readings were measured at $OD450/_{490\ nm}$ using a microtiter plate reader (Synergy HTX Multi-Mode Reader, Bio-Tek Instruments Inc.). The absorbance was expressed as a percentage relative to the control.

2.3.2 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay measures mitochondrial reductive capacity, which serves as an indirect measurement of cell viability and cell proliferation. For a MTT assay, following the treatment protocol previously described (**Figure 2.1-A**), cells were incubated with the MTT reagent. MTT was prepared by dissolving MTT powder (Sigma Aldrich, Cat # M2003) in phosphate buffered saline (PBS) to a concentration of 0.01 g/mL and further diluted to a 1:3 ratio and warmed to 37°C in preparation. After the respective treatment period, the treatments were removed, each experimental well rinsed with PBS and treated with 300 µL of MTT solution. The plate was subsequently transferred to the incubator for 90 minutes. MTT is converted to insoluble formazan crystals in proportion to the degree of metabolic activity. After incubation, the MTT solution was removed and 300 µL of Isopropanol/Triton-X solution (50 mL Acidic Isopropanol (99 mL isopropanol + 1 mL HCl); 1 mL 1% Triton-X), which dissolves the crystals, was added to each experimental well and additional blank wells. The plates were briefly shaken on a shaker for 5 minutes, and readings were measured at $OD595/_{490\ nm}$ using a microtiter plate reader (Synergy HTX Multi-Mode Reader, Bio-Tek Instruments Inc.). The absorbance was expressed as a percentage relative to the control.

2.4 Morphological Imaging

Phase contrast imaging allows the assessment of possible morphological changes induced by treatment. Following the treatment protocol depicted in **Figure 2.1-A**, a Zeiss Olympus® CKX31 (Olympus®, GMBH Japan) inverted microscope equipped with an Axiocam 208 color camera and Zeiss Laboscope software (Carl Zeiss, Germany) was used to acquire brightfield images at 4x and 10x magnification.

2.5 Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was used to assess messenger RNA (mRNA) levels. It requires RNA extraction and the conversion of RNA to DNA, allowing qPCR to be executed with the use of primers for a target of interest. A brief overview has been depicted in **Figure 2.2**.

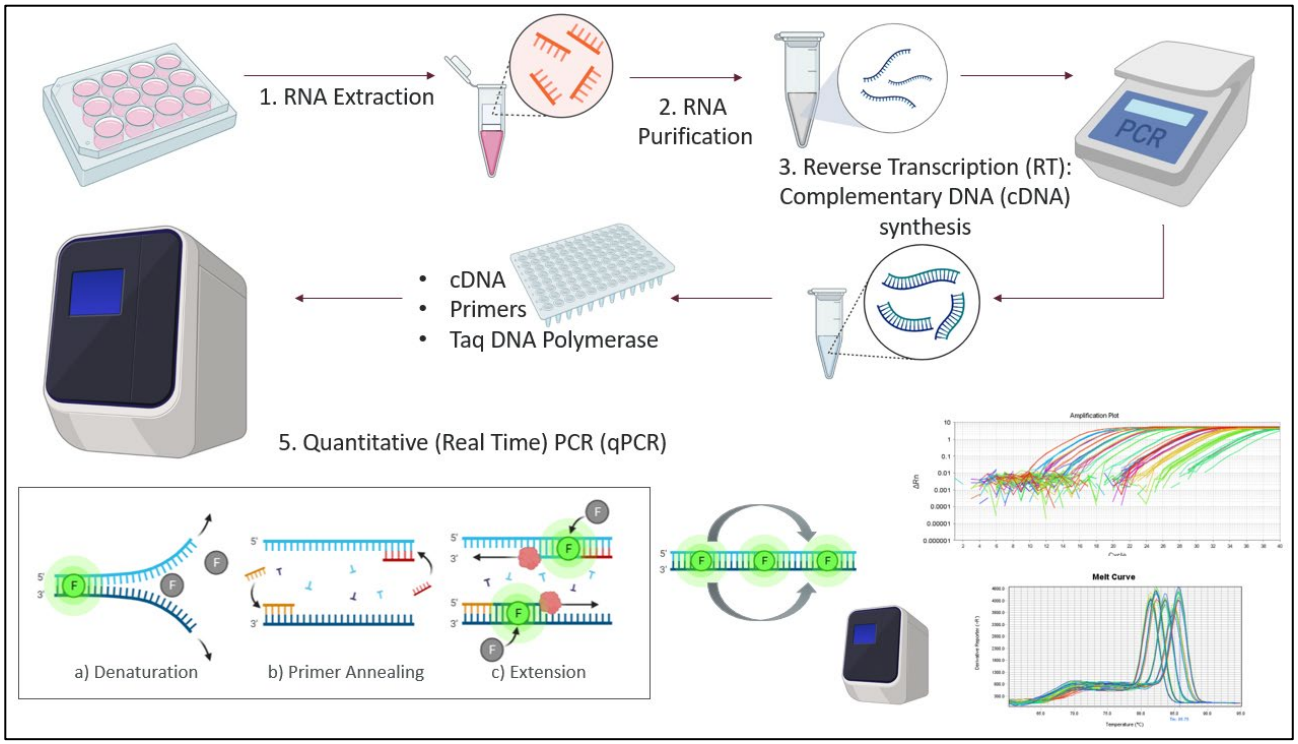

Figure 2.2: A brief overview of RT-qPCR protocol. (Created in Biorender.com). Abbreviations: PCR, Polymerase chain reaction; RNA, Ribonucleic acid.

2.5.1 RNA extraction

RNA was extracted with a protocol based on ThermoFisher Scientific's protocol for their TRIzol™ Reagent (Gibco™, Cat # 15596026). In a sterile and nuclease-free environment, following the treatment protocol previously described (**Figure 2.1-A**), the growth medium was removed and a pre-calculated amount of TRIzol™ reagent was added directly to the cells. The reagent was aspirated over the cells to ensure homogenization and left to incubate for 5 minutes. Samples were subsequently transferred to microcentrifuge tubes, followed by the addition of 0.2 mL Chloroform (Sigma-Aldrich, Cat # C2432-500) per 1 mL TRIzol™ used for each treatment group, followed by a 3-minute incubation step.

Next, samples were centrifuged at 4°C for 15 minutes at 12 000 g. This was followed by careful collection and transfer of the aqueous phase, which contains the RNA, to a new microcentrifuge tube. After the addition of 0.5 mL Isopropanol per 1 mL TRIzol™ used, samples were incubated for 10 minutes at room temperature and subsequently centrifuged for 10 minutes at 12 000 g. The supernatant was removed, the pellet was resuspended in 1 mL 75% Ethyl Alcohol (EtOH), briefly vortexed and centrifuged for 5 minutes at 7500 g.

The supernatant was then removed and the pellet airdried for 5-10 minutes, while monitoring to ensure it does not dry-out. Once dried, the RNA was suspended in 25-50 µL RNase-free dH_2O and incubated for 12 minutes at 57°C. The RNA yield and quality was determined using a Nanodrop LITE (Spectrophotometer, ThermoFisher Scientific), and samples were aliquoted and stored at -80°C until further analysis. RNA quality was confirmed with a 1% agarose gel and the presence of the 28S and 18S ribosomal RNA bands.

2.5.2 Primer design & polymerase chain reaction (PCR) quality check

Primers were chosen from literature (MDR1, LOX and Biglycan) or designed by Atarah Rass (VEGFR2). Primers were analyzed using the OligoAnalyzer™ Tool (Integrated DNA Technologies (IDT)) and PRIMER-blast tool (National Centre for Biotechnology Information). Primers were purchased from and prepared according to IDT recommendations in TE (Tris-EDTA) buffer (pH 8, Sigma- Aldrich, Cat # 93283-100ML) and nuclease-free water.

Amplification of the genes of interest was achieved with the following primers spanning exon-exon boundaries: **VEGFR2** (NM_002253.4) forward: AGCAGGATGGCAAAGACTAC, reverse: TACTTCCTCCTCCTCCATACAG (amplicon length, 116 bp); **MDR1** (All transcripts: NM_001348945.2, NM_001348944.2, NM_000927.5, NM_001348946.2) forward: TGACAGCTACAGCACGGAAG, reverse: TCTTCACCTCCAGGCTCAGT (amplicon length, 131 bp)

(Haque et al., 2020); **LOX** (All transcripts: NM_002317.7; NM_001178102.2, NM_001317073.1) forward: CCAGAGGAGAGTGGCTGAAG, reverse: CCAGGTAGCTGGGGTTTACA (amplicon length, 224 bp) (Adamopoulos et al., 2016); **Biglycan** (NM_001711.6) forward: AGGAGGCGGTCCATAAGAAT, reverse: AGGGTTGAAAGGCTGGAAAT (amplicon length, 110 bp) (Yamamoto et al., 2012). The reference genes, HPRT1 and RPLP0, were used to normalize quantitation cycle values.

To check primer functionality and evaluate the optimal primer concentration, routine PCR was conducted according to the manufacture's protocol for TaqMan® 2X Master Mix with Standard Buffer (New England Biolabs® Inc.). All individual components were added to PCR and gently mixed via aspiration, while working on ice. Samples were incubated in a preheated thermocycler using the recommended thermocycling conditions. The optimal melting temperature was determined through optimization and primer recommendations.

2.5.3 Reverse transcription: complementary DNA (cDNA) synthesis & quantitative polymerase chain reaction (qPCR)

The removal of potential genomic DNA (RNA purification) was done using DNase I, RNase-free kit (ThermoFisher Scientific), according to the manufacturer's protocol. DNA-free samples were stored at -20°C or used for primer optimization and cDNA synthesis. cDNA (5 ng) was synthesized using LunaScript™ RT Supermix Kit (New England Biolabs® Inc.), according to the manufacturers protocol. Once completed, samples were stored at -20°C or used in PCR or qPCR reactions. The qPCR reactions were conducted according to Luna® Universal qPCR Master Mix (New England Biolabs® Inc.) protocol. cDNA was diluted and primer-specific qPCR master mixes were prepared and subsequently loaded into a qPCR plate. Reactions were conducted with a StepOnePlus instrument (Applied Biosystems, Waltham, MA) and gene expression was quantified with the delta-delta quantitation ($2^{\Delta\Delta Ct}$) cycle method.

2.6 Western Blotting

Western blots were used to assess the protein expression of our markers of interest. It involves protein extraction, protein determination and sample preparation, SDS-PAGE (Sodium dodecyl-sulphate polyacrylamide gel electrophoresis), membrane transfer, antibody probing and chemiluminescent imaging, as depicted in **Figure 2.3**.

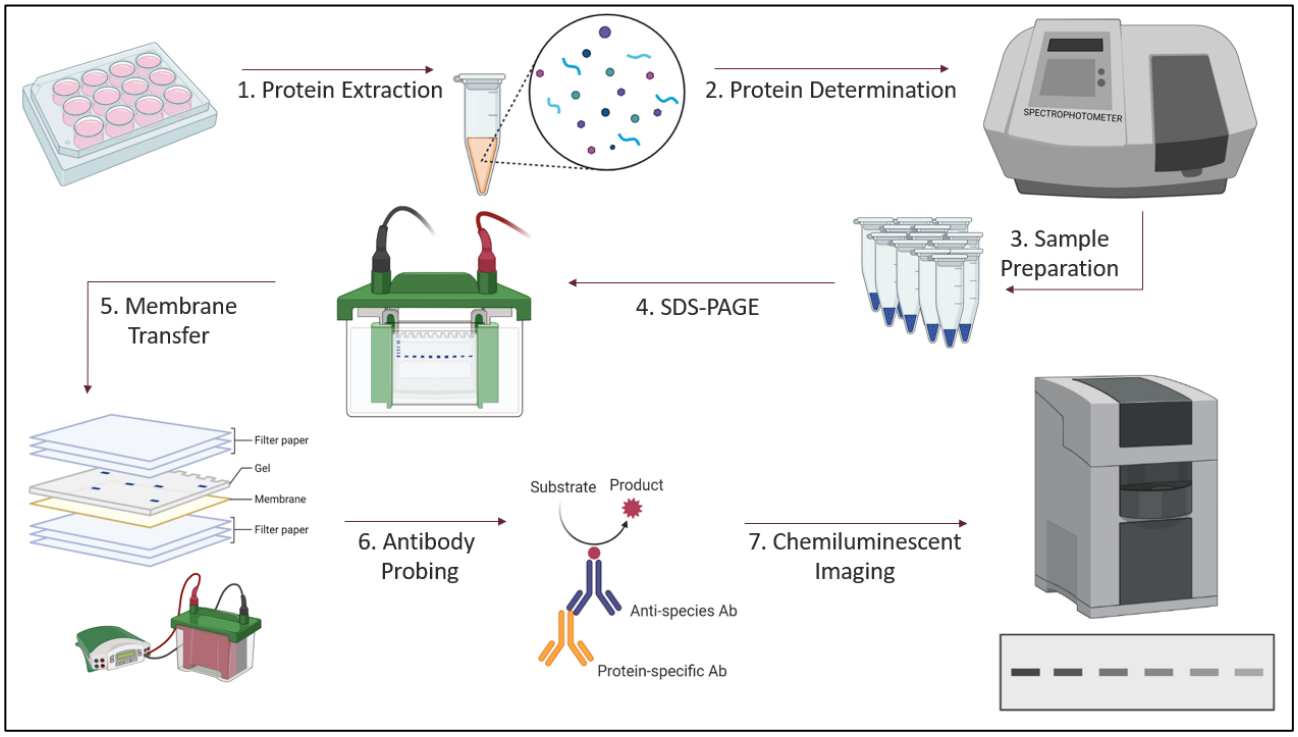

Figure 2.3: A brief overview of a western blot protocol. Abbreviations: SDS-PAGE, Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (Created in Biorender.com).

2.6.1 Protein Harvest

Following the treatment protocol previously described (**Figure 2.1-A**), the cells were placed on ice, the supernatant removed, and the cell monolayer rinsed twice with ice cold PBS. The cells were subsequently incubated with cold modified Radioimmunoprecipitation assay (RIPA) buffer for 1 hour, after which the cells were scraped and transferred to a pre-cooled microcentrifuge tube. The samples were then stored at -80°C until further analysis.

2.6.2 Cell Lysate Preparation, Protein Determination and Sample Preparation

The samples were thawed and centrifuged at 11 000 rpm, for 2 minutes at 4°C. The supernatant was transferred to a precooled microcentrifuge tube, generating whole cell lysates.

A Bradford assay was utilized to determine the concentration of protein in the cell lysates. The Bradford Assay generates a standard curve based on the absorbance values of standard samples (Bradford, 1976). The standard samples were prepared using varying dilutions of 2 mg/mL bovine serum albumin (BSA) samples diluted with dH_2O. The Bradford samples were prepared using 5 µL of cell lysates diluted with dH_2O (1:10). All samples were prepared in duplicate.

Bradford working solution (900 µL) was added to each sample, followed by vortexing and a 10-minute incubation period. The absorbances were subsequently measured at 595 nm using a Cecil CE 2021 (2000 Series) spectrophotometer. The standard curve was generated, and each sample was compared to the curve to assess protein concentration and the required volumes using a Microsoft Excel sheet.

Sample aliquots of 25 µg protein were prepared following protein determination. Cell lysates were diluted to a 2:1 ratio of cell lysate to Laemmli's sample buffer (850 µL sample buffer and 150 µL β-mercaptoethanol) and stored at – 80°C until further use. A detailed protocol for the Bradford assay has been described in **Appendix D**.

2.6.3 SDS-PAGE (Sodium dodecyl-sulphate polyacrylamide gel electrophoresis) and Western Blot

Once protein samples were thawed on ice, samples were dry boiled at 95°C for 5 minutes, pulsed and returned to the ice. Gel electrophoresis was used for protein separation with 12% gels (TGX Stain-Free™ FastCast™ Acrylamide kit, Biorad) and the Bio-Rad Power Pac 300. Each gel was loaded with 4 µL of the protein marker ladder, used to determine the molecular weights of the different protein bands, and 25 µg of protein per sample. Gels initially ran at 80 V for approximately 10-20 minutes, then at 100 V for approximately 75 minutes. Immediately after protein separation was completed, gels were activated in the Bio-Rad Chemidoc MP Imaging System.

Proteins were subsequently transferred to nitrocellulose membranes (Transblot Turbo™ RTA Transfer Kit, Nitrocellulose BioRad) using the Transblot Turbo Transfer System (Bio-Rad). The membranes were imaged for total protein and blocked in 5% fat-free milk with TBS-T (1X Tris-Buffered Saline, 0.1% Tween® 20) for 1 hour or in Bio-Rad Blocking Buffer for 5 minutes at room temperature. Membranes were washed three times for 5 minutes with TBS-T and incubated overnight in primary antibodies at 4°C. The following primary antibodies were utilized: TEM7 (ThermoFisher Scientific, MA141065); TEM8 (ThermoFisher Scientific, MA191702); CXCR7 (Sigma-Aldrich (Pty), SAB4502446) and Mini-chromosome maintence-2 (MCM2) (Cell Signalling, #4007), listed in **Table 2.3**.

Table 2.3: Primary antibodies used to assess a tumour endothelial cell phenotype and cell proliferation.

Antibody	Species	Concentration	Size (kDa)
Tumour endothelial cell markers			
TEM7	Mouse	1:1000	60
TEM8	Mouse	1:10 000	45
CXCR7	Rabbit	1:1000	50
Proliferation marker			
MCM2	Rabbit	1:1000	102

Following primary antibody incubation, membranes were washed three to five times in TBS-T for 5-10 minutes - relative to the protein of interest. The membranes were subsequently incubated in horseradish peroxidase-conjugated secondary antibodies (1:10000 dilution) for 1 hour at room temperature. This was followed by washing in TBS-T (three x 5 minutes) and subsequent imaging using the Chemidoc. Imaging required an enhanced chemiluminescence substrate (Clarity Western ECL Substrate, Cat # 1705061) prepared in a 1:1 ratio of substrate to enhancer. Imaging and analyses were done using Image Lab software™ (Image Lab 6.0.1 Software, Biorad).

2.7 Scratch Assay

2.7.1 Mitomycin C (MMC) dose response

MMC inhibits DNA synthesis and is used to exclude any effects of cell proliferation. It was purchased from Sigma-Aldrich (Cat # M4287) and prepared as a stock solution of 0.4 mg/mL in sterile PBS, which was aliquoted and stored at 4°C in the dark, as MMC is light sensitive. Optimal concentrations of MMC to inhibit cell proliferation without inducing cell death was determined with a dose response experiment for the HUVEC cell line. The three concentrations of MMC (1 µg/mL, 5 µg/mL and 10 µg/mL) were prepared in culture medium (MMC medium) based on the use of MMC with HUVECs in literature. It was determined that a concentration of 5 µg/mL MMC was sufficient for the inhibition of cell proliferation over a 24-hour treatment period.

Cells were seeded on sterile coverslips in 6-well plates, incubated for 24 hours and treated for 24 hours with culture medium (24-hour control) or MMC medium. A 0-hour control group was fixed and stained at the time of treatment. After the 24-hour period, the remaining groups were fixed and stained. Cells were fixed with 1:1 10% Neutral Buffered Formalin (NBF) (Leica Biosystems Richmond, Inc., Cat # 3800604EG) and treatment medium for 5 minutes, followed by a 5 minute

10% NBF only incubation. The coverslips were rinsed three times with PBS and subsequently stained with the nuclear dye Hoescht (1:200 Hoescht and PBS) for 10 minutes. Nuclear staining was performed in the dark as Hoescht is light sensitive. The coverslips were rinsed three times with PBS, mounted with DAKO Fluorescent Mounting Medium onto microscope slides and left to dry for 1 hour at room temperature. Clear nail polish was used to seal the slides, which were dried for 1 hour at room temperature and stored at -20°C.

Nine images were captured at random fields of view per biological repeat (n=3) on a Nikon Eclipse E400 microscope equipped with a DS-12 colour digital camera (Nikon, Japan). The nuclear counts, which were obtained using ImageJ Software, were recorded and the 24-hour groups were compared to the 0-hour control (**Table 2.4**).

Table 2.4: Nuclear counts of HUVECs for MMC dose response.

Treatment group (time point)	**Control**		**MMC (24-hour)**		
	0-hour	24-hour	1 µg/mL	5 µg/mL	10 µg/mL
Average count (n=9)	**482,07**	903,04	784,33	**563,70**	500,56
Total count	**4186,67**	8395,67	6346,67	**4502,67**	3788,33

2.7.2 Scratch assay

A scratch assay, or a wound healing assay, mimics *in vivo* wound healing and measures the migratory activity of cells. To assess cell migration, a scratch was made to a confluent cell monolayer with a SPLScar Scratcher (Bio-Smart), following the treatment protocol described in **Figure 2.1-B**. Cell debris was removed by rinsing with warm PBS and fresh MMC-supplemented treatment medium was added. Positions were established on top of the cell culture plate with a permanent marker to ensure that the exact position in the wound is monitored over time. Initial images were captured at the 0-hour time point (T0), followed by re-imaging at 6- (T6), 12- (T12) and 24- (T24) hour time points. Bright field images were acquired with a Zeiss Olympus® CKX41 inverted microscope (Olympus®, GMBH Japan) equipped with an Axiocam 208 color camera and Zeiss Laboscope software (Carl Zeiss, Germany), and analyzed using ImageJ Software.

The percentage wound closure was calculated using the following formula:

$$\frac{wound\ area\ (0\ hour) - wound\ area\ (x\ hour)}{wound\ area\ (0\ hour)} \times 100 = \%\ wound\ closure\ at\ x\ hour$$

The rate of wound closure was calculated using the following formula:

$$\frac{\%\ wound\ closure\ (x\ hour)}{x} = rate\ of\ wound\ closure\ at\ x\ hour\ (\%.hour^{-1})$$

2.8 Tube formation assay

A tube formation assay relies on the use of basement membrane matrix that mimics the *in vivo* environment of endothelial cells. Endothelial cells therefore form tube-like structures of elongated cells and lumens surrounded by linked endothelial cells when seeded on a basement membrane in the presence of angiogenic inducers (DeCicco-Skinner et al., 2014).

Cells were seeded from thawing and culture medium was replaced every 2-3 days until confluent. Basement membrane matrix (Geltrex, Thermofisher Scientific®, Cat # A1413202) was seeded in pre-cooled 24-well plates (100 µL/well) using pre-cut p200 pipette tips and incubated for 30 minutes. Cells were seeded in non-supplemented medium and treated at time point 0 according to the protocol depicted in **Figure 2.1-B**. The cells were imaged over a desired period using the Olympus Provi CM20 incubation monitoring system (Olympus Life Science, Japan), as illustrated in **Figure 2.4**, and subjected to qualitative assessment.

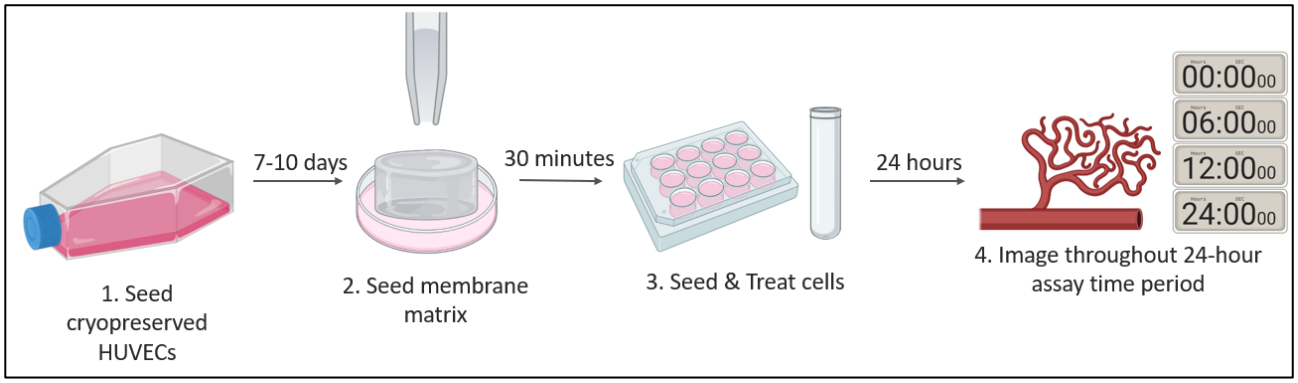

Figure 2.4: A brief overview of a tube formation assay protocol. (Created in Biorender.com). Abbreviations: HUVECs, Human umbilical vein endothelial cells.

2.9 Statistical analyses

The graphic results are represented as Mean ± Standard Error of the Mean (SEM). Pertaining to RT-qPCR data, StepOne™ v2.3 software was used to normalize and obtain the data, all values are expressed as $2^{-\Delta\Delta Ct}$, for the relative quantification of gene expression (Livak & Schmittgen, 2001). Pertaining to western blot data, Image Lab software™ was used for normalization of the controls and to obtain the data, all values were expressed as a percentage of the control. All statistical analysis was done using GraphPad Prism® 7 for Windows® (GraphPad, San Diego, CA). The relevant statical tests are displayed in **Table 2.5** and significance was set at $p<0.05$.

Table 2.5: Statistical tests used to analyze data of different assays.

Experiments	Statistical Tests
Cell viability (WST-1) Gene expression (RT-qPCR) Protein expression (western blots)	One-way analysis of variance (ANOVA) Tukey post-hoc/Fishers LSD
Scratch Assay	Two-way ANOVA Sidak post-hoc/Fishers LSD

Chapter 3: Results

3.1 Establishing a tumour endothelial cell (TEC) phenotype in breast cancer

TECs function as defective hyperactivated endothelial cells with structural and functional abnormalities. Although, the roles of TECs in cancer progression are vast, they provide sustained angiogenesis, routes for intravasation, tumour growth through paracrine signalling, inflammatory responses and they also contribute to treatment resistance (Bussolati et al., 2003; Dudley et al., 2008; Fessler et al., 2015; Ingthorsson et al., 2010; Maishi et al., 2016; Nagl et al., 2020; Sigurdsson et al., 2011). TECs may also serve as targets in anti-angiogenic therapy with expression profiles thought to differ from their normal counterparts (Kopczyńska & Makarewicz, 2012). However, information pertaining to how breast cancer influences endothelial cells and the TEC phenotype is limited, and how the changes differ in response to non-malignant versus malignant signalling is unknown. An *in vitro* model was used to establish and characterize a TEC phenotype in breast cancer. Additionally, a comparative model of the response to non-malignant and malignant signalling was created with the use of MCF-12A, MCF-7 and MDA-MB-231 cell lines. CM was harvested from breast cells and used to treat HUVECs for 24 hours, followed by subsequent assessment.

3.1.1 Cell viability

To assess the effect of breast cell CM on the cell viability of endothelial cells, a WST-1 assay, which measures mitochondrial reductive capacity, was used. An increase in mitochondrial reductive capacity is an indication of increased in metabolic activity and is therefore associated with an increase in cell viability and cell proliferation. Following dose response tests with CM from supplemented breast cells (**Supplementary Figure S2.1**) and tests with the complete culture medium of the breast cells (**Supplementary Figure S2.2**), it was concluded that some of the effects observed may be as a result of remaining supplements present in the CM.

Breast cells were then treated with culture medium free of growth factors and supplements, whereafter CM was collected. HUVECs were treated with 100% starved (no supplements) CM for 24 hours. As depicted in **Figure 3.1**, compared to the control (100% ± 0.55%), the cell viability of all the breast CM groups significantly increased ($p<0.0001$). Significant differences in cell viability are noted between the non-malignant MCF-12A CM group (267.9% ± 1.45%) and both the breast cancer CM groups ($p<0.0001$), and between the malignant MCF-7 (309.2% ± 2.9%) and MDA-MB-231 (284.9% ± 3.9%) CM groups ($p<0.0001$).

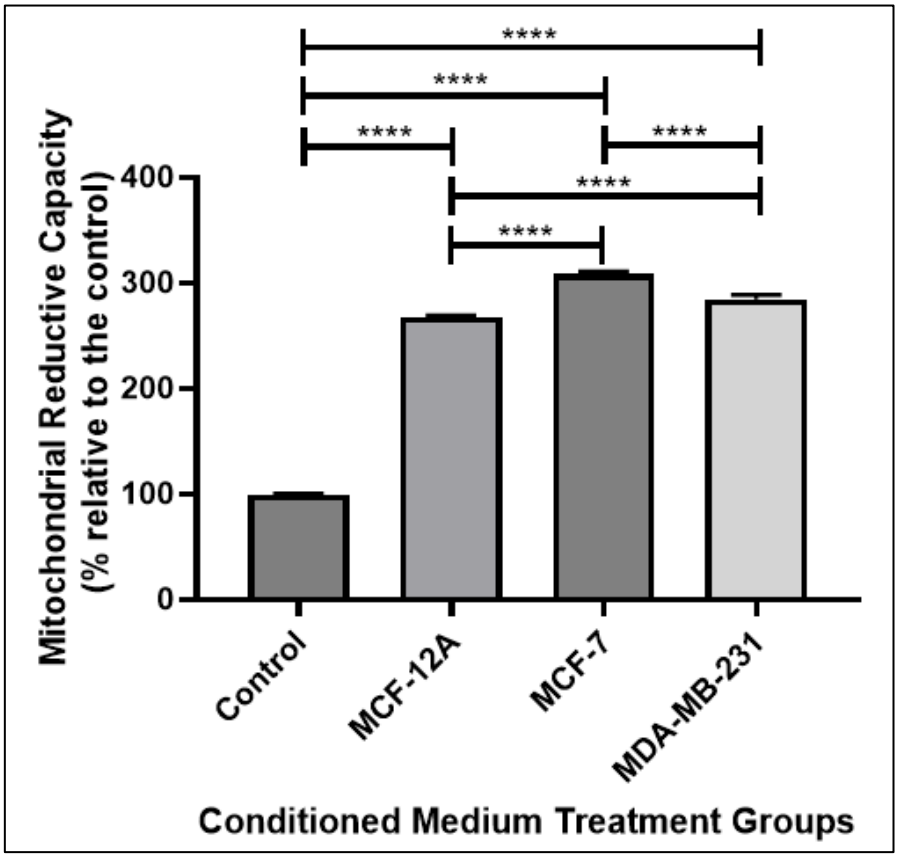

Figure 3.2: Mitochondrial reductive capacity as an indirect measure of cell viability (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were treated with 100% CM for 24 hours and subjected to a WST-1 assay. Significant differences were observed between all CM groups. Results are expressed as mean ± SEM. Asterisks (****) denotes $p<0.0001$. Abbreviations: HUVECs, Human umbilical vein endothelial cells; WST-1, Water-soluble tetrazolium salt-1.

3.1.2 Cell morphology

Signalling from the microenvironment influences endothelial cell morphology that either is required for angiogenic activity and/or accompanies a dysfunctional phenotype (Dudley, 2012; Mcdonald & Choyke, 2003). Phase contrast imaging was used to assess changes in the morphology of HUVECs induced by CM from non-malignant and malignant breast cells. Qualitatively, microscopic analysis revealed cell elongation (red arrows), lumen formation (hollow spaces) (blue circles) and a form of branching (yellow arrows), which occurred to a greater extent in HUVECs treated with CM from breast cancer cells and more in the MDA-MB-231 CM group compared to the MCF-7 CM group (**Figure 3.2**).

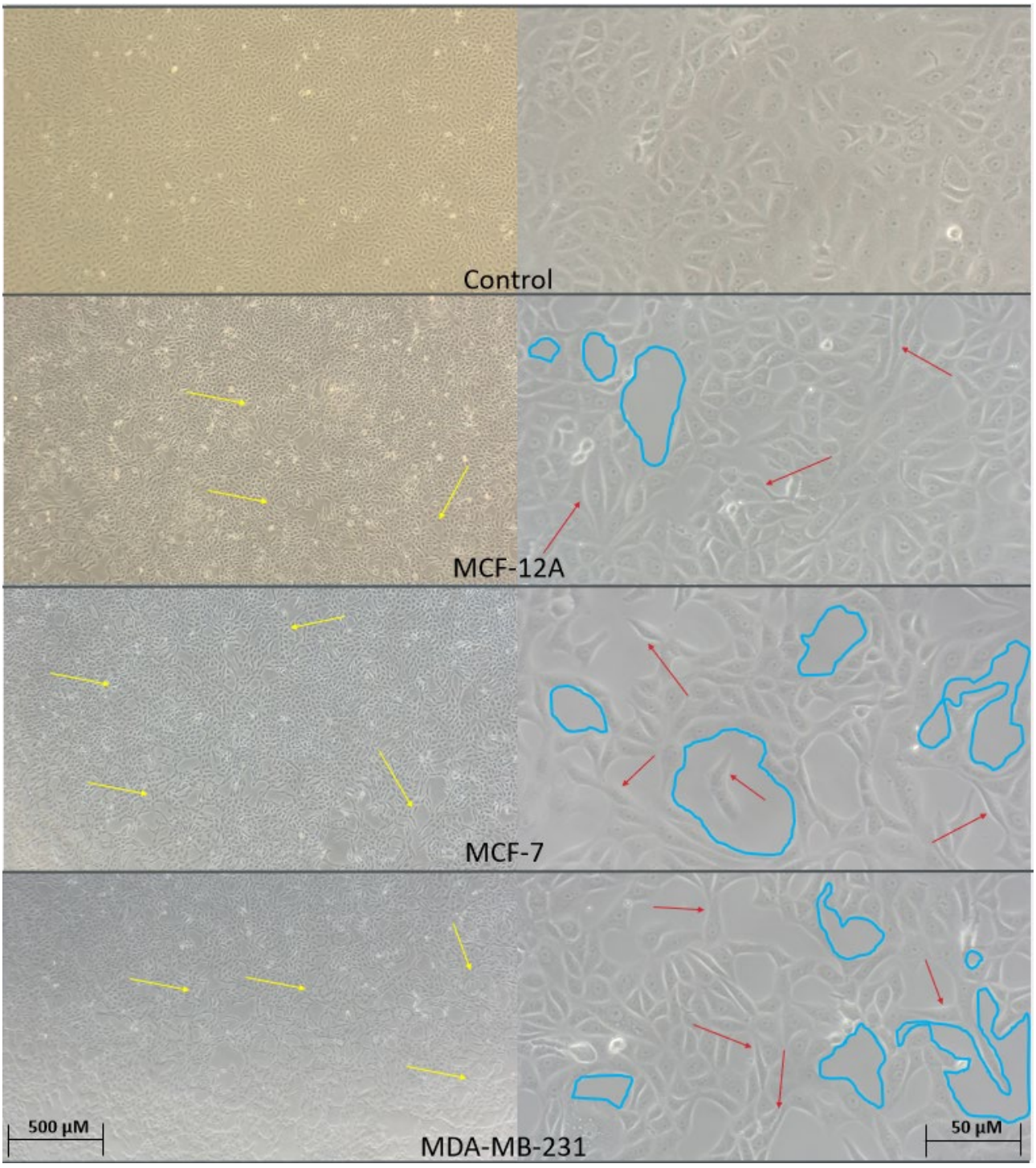

Figure 3.2: Phase contrast imaging to assess changes in cell morphology of HUVECs following conditioned media treatment (n=2). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were treated with 100% CM for 24 hours and imaged to assess changes in cell morphology induced by breast CM groups. Cell elongation is indicated with red arrows, lumens with blue circles and branching with yellow arrows. Brightfield images were acquired with a Zeiss Olympus® CKX31 (Olympus®, GMBH Japan) inverted microscope equipped with an Axiocam 208 color camera and Zeiss Laboscope software (Carl Zeiss, Germany) at 4x (500 µM) and 10x (50 µM) magnification. Abbreviations: HUVECs, Human umbilical vein endothelial cells.

3.1.3 Gene expression

Crosstalk with tumourigenic cells and crosstalk with the local TME induces genetic changes in TECs responsible for their abnormal pro-tumourigenic features (Hida, Hida & Shindoh, 2008; Hotchkiss et al., 2005; St. Croix et al., 2000). RT-qPCR was used to evaluate the relative gene expression of markers VEGFR2, MDR1, Biglycan and LOX, associated with the TEC phenotype.

3.1.3.1 VEGFR2

VEGFR2 is a cell surface receptor for VEGFs and acts as the predominant receptor during angiogenesis. It mediates various angiogenic processes and serves as a marker of cell proliferation and survival (Wang et al., 2020; Karaman, Leppänen & Alitalo, 2018). VEGFR2 expression significantly decreased in all CM groups (MCF-12A: 0.45 ± 0.14, $p<0.01$; MCF-7: 0.27 ± 0.03, $p<0.001$; MDA-MB-231: 0.16 ± 0.07, $p<0.001$) compared to the control (**Figure 3.3.1**).

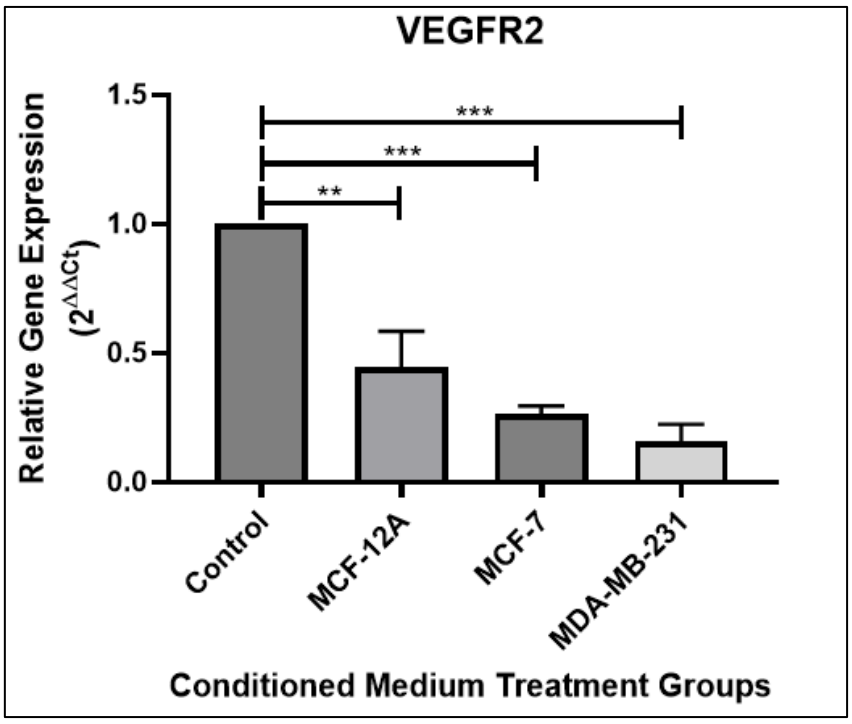

Figure 3.3.1: RT-qPCR analysis to detect VEGFR2 in HUVECs following conditioned media treatments (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were subsequently treated with 100% CM for 24 hours, RNA was extracted and used for analysis**.** Significant decreases were observed between the control and all the CM groups. Vertical bars denote mean ± SEM (n=3). Asterisks (**, ***) denotes $p<0.01$ & $p<0.001$, respectively. Abbreviations: HUVECs, Human umbilical vein endothelial cells; RNA, Ribonucleic acid; VEGFR2, Vascular endothelial growth factor receptor 2.

3.1.3.2 MDR1

MDR1 encodes P-glycoprotein, a protective efflux pump that removes molecules present within the cell or cell membrane. Cells acquire MDR1 expression through (1) general physiological expression, (2) proliferation pathway activation and stress responses during conditions such as hypoxia, and (3) exposure to cytotoxic drugs (Katayama, Noguchi & Sugimoto, 2014; Huang et al., 2013; Comerford, Cummins & Taylor, 2004; Régina et al., 2001). MDR1 expression significantly increased in the MCF-12A CM group (1.84 ± 0.10) compared to the control ($p<0.001$) and MCF-7 (1.02 ± 0.03) ($p<0.001$) and MDA-MB-231 (1.03 ± 0.13) ($p<0.001$) CM groups (**Figure 3.3.2**).

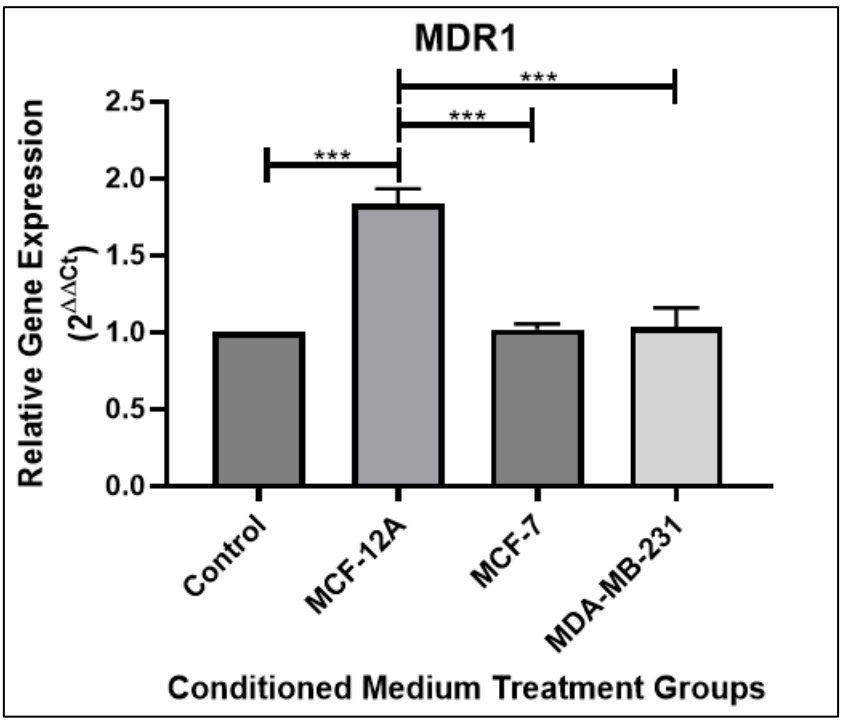

Figure 3.3.2: RT-qPCR analysis to detect MDR1 in HUVECs following conditioned media treatments (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were subsequently treated with 100% CM for 24 hours, RNA was extracted and used for analysis**.** A significant increase was observed in the MCF-12A CM group, compared to the control and MCF-7 CM group. Asterisks (***) denotes $p<0.001$. Abbreviations: HUVECs, Human umbilical vein endothelial cells; MDR1, Multidrug resistance 1; RNA, Ribonucleic acid.

3.1.3.3 Biglycan

Biglycan is a proteoglycan that serves as a structural component of the extracellular matrix (Nastase, Young & Schaefer, 2012). Biglycan is a pro-angiogenic protein that is undetectable under normal conditions (Hu et al., 2016; Berendsen et al., 2014; Obika et al., 2013; Calabrese et al., 2011). No

significant differences were observed in Biglycan expression between any of the CM groups ($p>0.05$) (**Figure 3.3.3**).

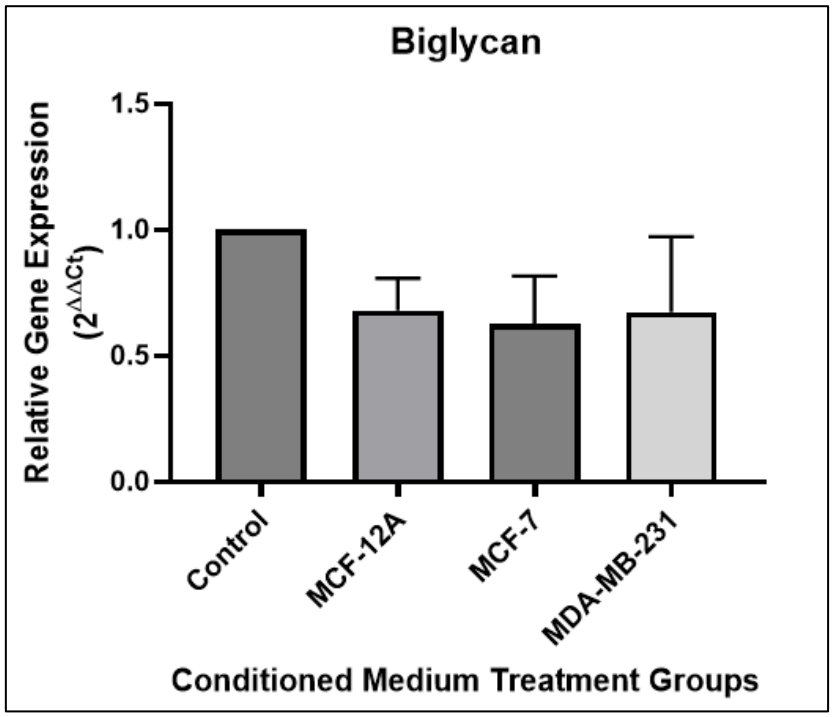

Figure 3.3.3: RT-qPCR analysis to detect Biglycan in HUVECs following conditioned media treatments (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were subsequently treated with 100% CM for 24 hours, RNA was extracted and used for analysis**.** No significant decreases were observed between any of the treatment groups. Vertical bars denote mean ± SEM (n=3). Abbreviations: HUVECs, Human umbilical vein endothelial cells; RNA, Ribonucleic acid.

3.1.3.4. LOX

LOX is an amine oxidase that serves as an ECM modifying enzyme (di Stefano et al., 2016; Smith-Mungo & Kagan, 1998). LOX expression, which possesses pro-angiogenic properties, is tightly regulated to maintain vascular homeostasis (Adamopoulos et al., 2016; Rodríguez et al., 2008). LOX expression was significantly increased in the MCF-12A CM group (2.02 ± 0.42) compared to control ($p<0.05$) and the MCF-7 CM group (1.03 ± 0.05) ($p<0.05$) (**Figure 3.3.4**).

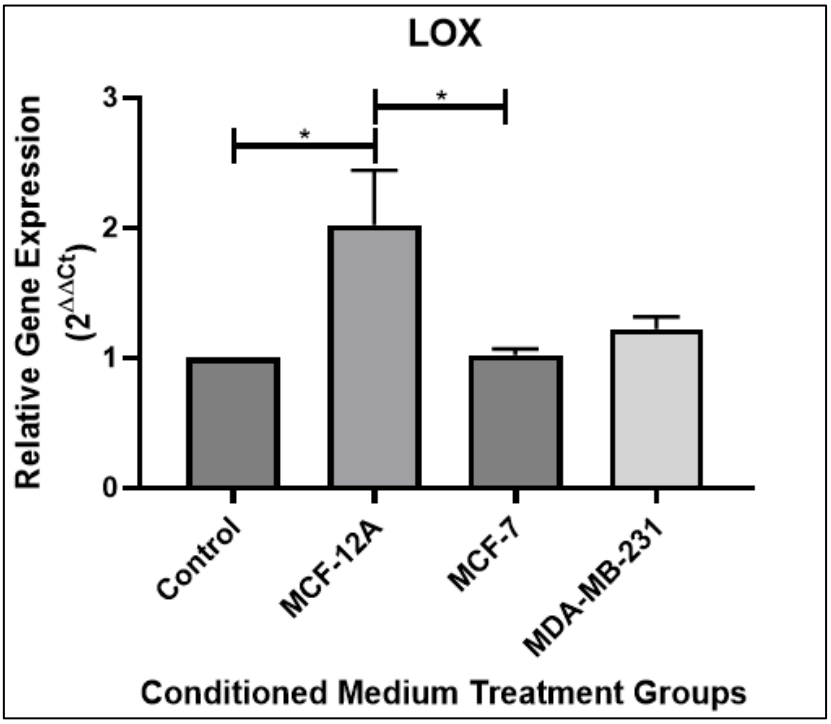

Figure 3.3.4: RT-qPCR analysis to detect LOX in HUVECs following conditioned media treatments (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were subsequently treated with 100% CM for 24 hours, RNA was extracted and used for analysis**.** A significant increase was observed in the MCF-12A CM group compared to the control and MCF-7 CM group. Vertical bars denote mean ± SEM. Asterisks (*) denotes p<0,05. Abbreviations: HUVECs, Human umbilical vein endothelial cells; LOX, Lysyl Oxidase; RNA, Ribonucleic acid.

3.1.4 Protein expression

Crosstalk with tumourigenic cells and crosstalk with the local TME prompt endothelial cells to express ligands and receptors that enable the cells to exert pro-tumourigenic functions (Hida, Hida & Shindoh, 2008; Hotchkiss et al., 2005; St. Croix et al., 2000). Western blots were used to assess the protein expression of TEM7, TEM8 and CXCR7, which are associated with a TEC phenotype; and MCM2, associated with proliferation.

3.1.4.1 TEM7

TEM7 is a cell surface protein that binds structural proteins, and is involved in cell adhesion, attachment and migration. TEM7 was named through the discovery that it was one of the genes overexpressed in tumour endothelium (St. Croix et al., 2000). No significant differences in TEM7 expression were observed between any of the CM groups (p>0.05) (**Figure 3.4.1**).

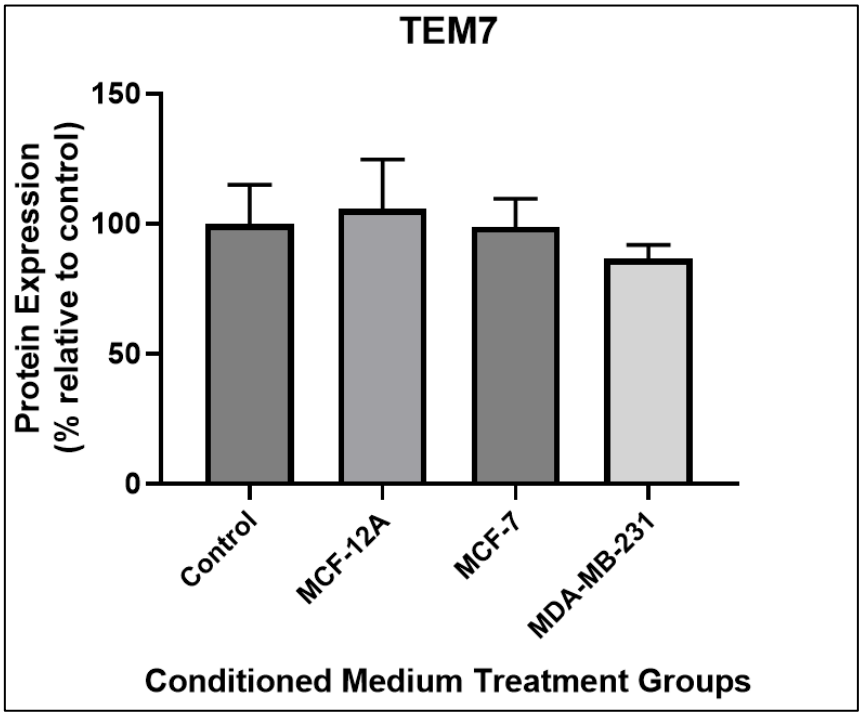

Figure 3.4.1: Western blot analysis to detect TEM7 in HUVECs following conditioned media treatments (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were subsequently treated with 100% CM for 24 hours, protein was extracted and used for analysis. No significant differences were observed between any of the treatment groups. Results are presented as mean ± SEM. Representative Western blot images can be found in **Figure 3.4.5**. Abbreviations: HUVECs, Human umbilical vein endothelial cells; TEM7, Tumour endothelial marker 7.

3.1.4.2 TEM8

TEM8 is a cell surface receptor known for embryonic angiogenesis and the binding of the anthrax toxin protein (Bonuccelli et al., 2005; Hotchkiss et al., 2005). TEM8 was named through the discovery that it was one of the genes overexpressed in tumour endothelium (St. Croix et al., 2000). The TEM8 (ANTXR1) is expressed as five splice variants that encode membrane bound and soluble receptors. Splice variant 1 encodes TEM8 membrane-bound receptor (85 kDa) and Splice Variant 3 encodes the extracellular domain of TEM8 (soluble receptor) (45 kDa) (Vargas et al., 2012; Hotchkiss et al., 2005).

Splice variant 1 and 3 were successfully detected as depicted in **Supplementary Figure S3**. It was, however, not possible to make a confident allocation of a band to the membrane bound TEM8 as it has not been allocated or quantified in literature (Hotchkiss et al., 2005). The secreted extracellular domain of TEM8 was, however, confidently allocated and used for protein quantification (**Figure 3.4.5**). As depicted in **Figure 3.4.2**, a significant increase in TEM8 expression was observed in the MCF-12A (177.9% ± 17.62%) ($p<0.05$) and MCF-7 (159.9% ± 21.59%) ($p<0.05$) CM groups

compared to the control (100% ± 15.26%). A significant difference was also observed between the MCF-12A and MDA-MB-231 (114.50 ± 22.05) CM groups ($p<0.05$).

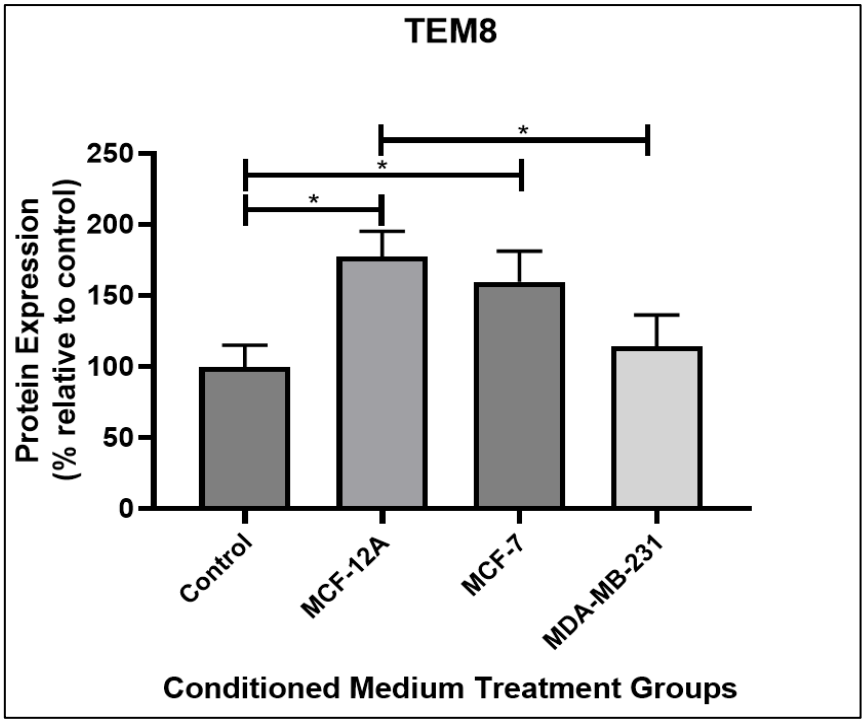

Figure 3.4.2: Western blot analysis to detect TEM8 in HUVECs following conditioned media treatments (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were subsequently treated with 100% CM for 24 hours, protein was extracted and used for analysis. Significant increases were observed in the MCF-12A and MCF-7 CM groups compared to the control, and in the MCF-12A CM group compared to the MDA-MB-231 group. Results are presented as mean ± SEM. Asterisks (*) denotes $p<0.05$. Representative Western blot images can be found in **Figure 3.4.5**. Abbreviations: HUVECs, Human umbilical vein endothelial cells; TEM8, Tumour endothelial marker 8.

3.1.4.3 CXCR7

CXCR7 is a scavenger receptor for CXCL12 and CXCL11 and a dimerization partner for CXCR4. It is associated with activated or transformed cells and has a wide range of roles, including contributing to angiogenesis and vasculogenesis (Adlere et al., 2019; Zhang et al., 2017; Maishi et al., 2012; Naumann et al., 2010). Although an increase was observed in the MCF-12A CM group (138.90% ± 9.96%) compared to the control (100% ± 14.72%) ($p=0.07$), it was not significant (**Figure 3.4.3**).

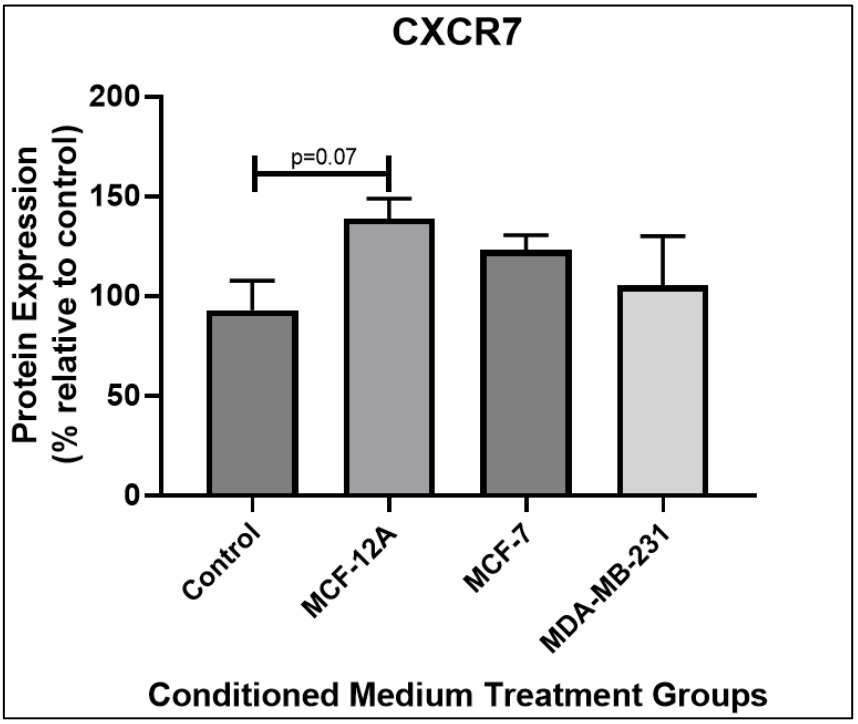

Figure 3.4.3: Western blot analysis to detect CXCR7 in HUVECs following conditioned media treatments (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were subsequently treated with 100% CM for 24 hours, protein was extracted and used for analysis. A non-significant increase was observed in the MCF-12A CM group compared to the control (p=0.07). Results are presented as mean ± SEM. Representative Western blot images can be found in **Figure 3.4.5**. Abbreviations: CXCR7, Chemokine CXC receptor 7; HUVECs, Human umbilical vein endothelial cells.

3.1.4.3 MCM2

MCM2 is part of a family of proteins involved in DNA replication and their activity serves as an indication of active cell proliferation (Nowińska & Dzięgiel, 2010). MCM2 was therefore used to assess the influence of breast CM on the proliferative activity of HUVECs. As depicted in **Figure 3.4.4**, no significant differences were observed between any of the treatment groups (P>0.05).

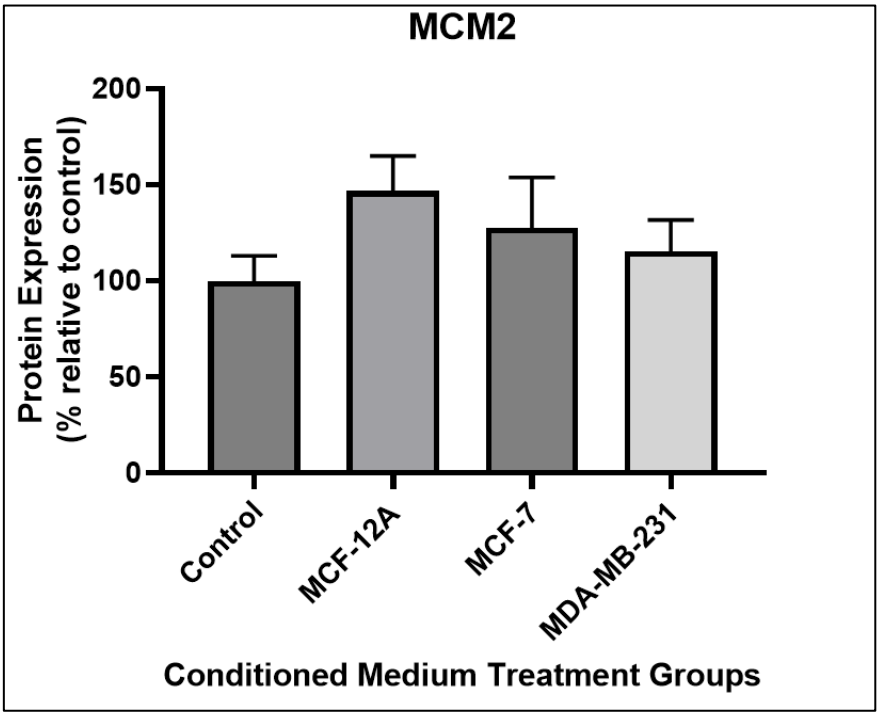

Figure 3.4.4: Western blot analysis to detect MCM2 in HUVECs following CM treatments (n=3). CM was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were subsequently treated with 100% CM for 24 hours, protein was extracted and used for analysis. No significant differences were observed between any of the treatment groups. Results are presented as mean ± SEM. Representative Western blot images can be found in **Figure 3.4.5**. Abbreviations: HUVECs, Human umbilical vein endothelial cells; MCM2, Mini-chromosome maintenance 2.

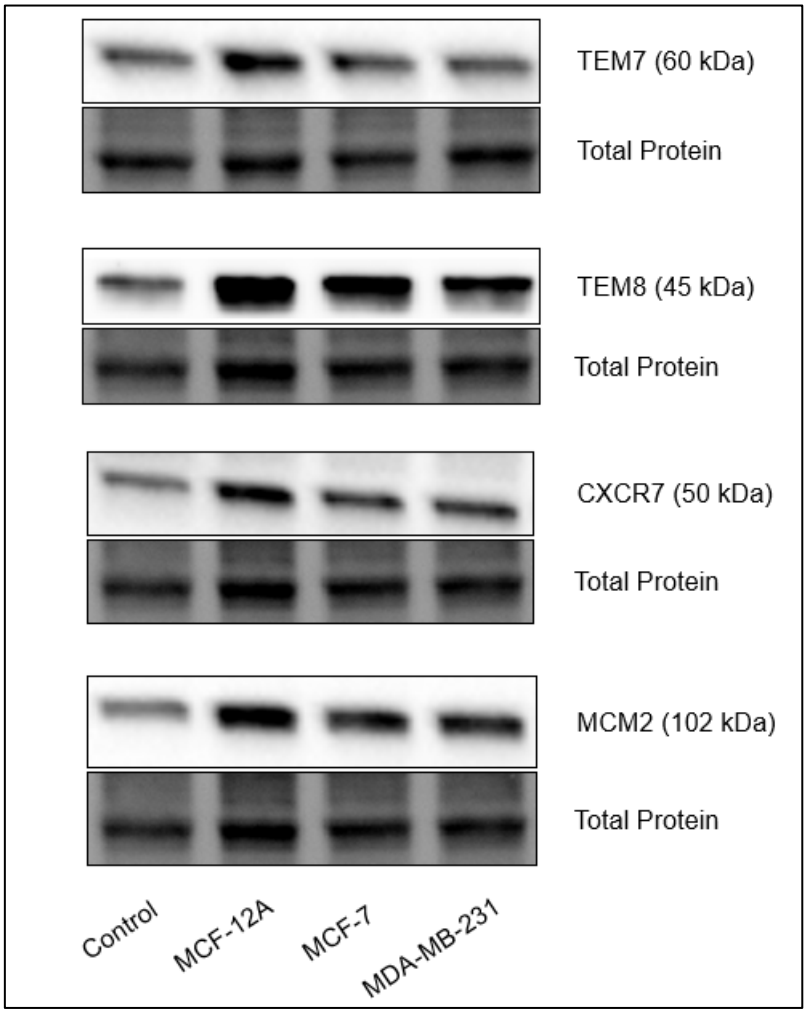

Figure 3.4.5: Representative images of Western blots for markers associated with a TEC phenotype and cell proliferation. SDS-PAGE was used for protein separation, followed by subsequent transfer to nitrocellulose membranes for analysis. Abbreviations: CXCR7, Chemokine CXC receptor 7; MCM2, Mini-chromosome maintenance-2; TEM7/8, Tumour endothelial marker 7/8.

3.2 Assessing the effect of paracrine signalling from breast cancer cells on the angiogenic processes

Inducing angiogenesis is a hallmark of tumours and is key to cancer progression for reasons such as tumour growth, invasion and metastasis (Frentzas, Lum & Chen, 2020; Hanahan & Weinberg, 2011). Cancer cells can elicit this response from endothelial cells by tipping the balance between pro-angiogenic and anti-angiogenic factors through the chronic release of a plethora of pro-angiogenic factors (Hanahan & Weinberg, 2011; Fox et al., 1993). Vessels generated by tumour angiogenesis are abnormal and inefficient, which also contributes to treatment resistance (Frentzas, Lum & Chen, 2020).

Angiogenesis is a multi-step process, involving various processes to establish a vascular network (Potente, Gerhardt & Carmeliet, 2011). *In vitro* analysis of angiogenesis is accomplished by mimicking conditions that prompt endothelial cells to respond according to their inherent abilities. An *in vitro* model was used to assess the influence of paracrine breast cell signalling on the angiogenic processes of cell migration and tube formation. A comparative model of a response to non-malignant and malignant signalling was created with the use of MCF-12A (control), MCF-7 and MDA-MB-231 cell lines. CM was harvested from starved breast cells and HUVECs were treated with 100% CM for 24 hours, followed by subsequent assessment. The control used to address the previous aim was used as a positive control, due to the positive response of the cells to their normal, supplemented culture medium. An angiogenic response was also expected in response to starvation so this model assesses how the response to breast cancer differs to the 'normal'/non-malignant response.

3.2.1 Cell migration

A scratch assay, also known as a wound healing assay, mimics cell migration during wound healing (Liang, Park & Guan, 2007). A scratch assay was used to assess the effect of breast cancer cells on the migratory activity of HUVECs. Dose responses revealed that the optimal dose of MMC, which inhibits DNA proliferation, for HUVECs was 5 µg/ml. Scratches were observed following MMC and CM treatment over a 24-hour period and representative images acquired at the 0, 6, 12 and 24-hour time points (**Figure 3.5.1 and 3.5.2**).

The 0-hour images were taken when the scratches were made and cells were treated, and the final images were taken at the end of the 24-hour treatment period. It was, however, very difficult to characterize the remaining wound (open space) at the 24-hour time points, especially for the MCF-7 CM group, which lead to the exclusion of the 24-hour time points from analysis.

As depicted in **Figure 3.5.1**, the MCF-7 CM group (31.05% ± 2.28%) elicited a significant increase in the percentage wound closure of HUVECs at 12 hours compared to the MCF-12A CM group (22.88% ± 3.39%) ($p<0.05$). The MCF-7 CM group (2.59%.hour^{-1} ± 0.19%.hour^{-1}) also exhibited an increase in the rate of wound closure at 12 hours compared to the MCF-12A CM group (1.91%.hour^{-1} ± 0.28%.hour^{-1}) ($p=0.08$), however, it was not significant.

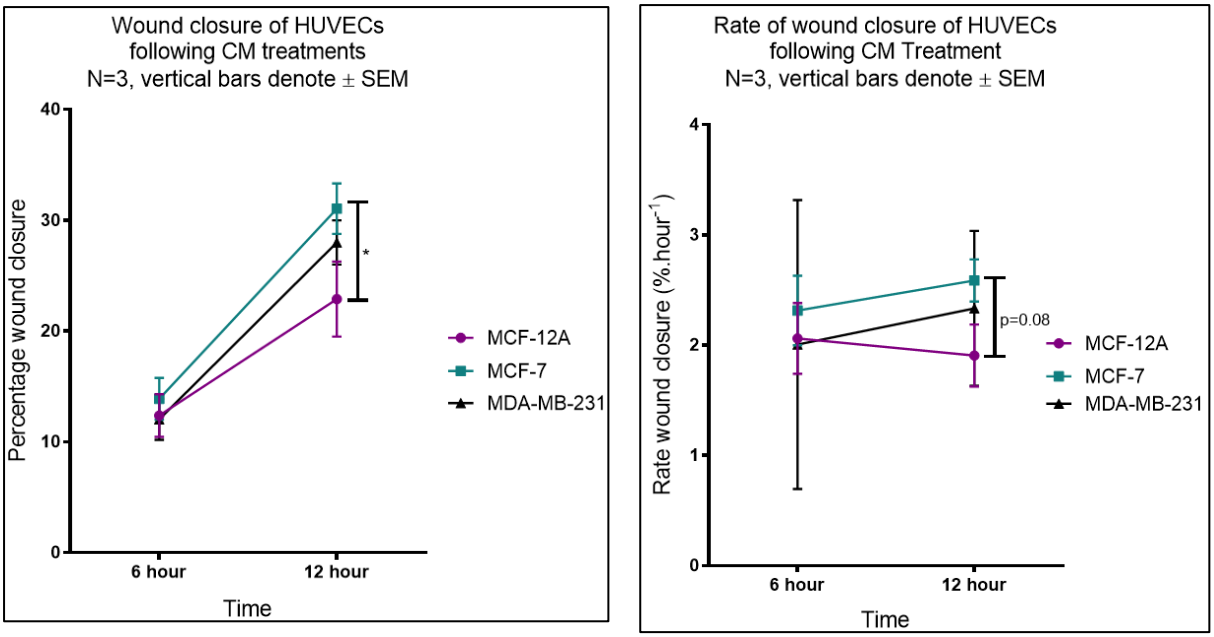

Figure 3.5.1: Percentage wound closure and rate of wound closure of HUVECs following conditioned media treatment (n=3). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were seeded, scratched, and treated with 100% CM for 24 hours. A significant difference was observed in the percentage wound closure of the MCF-7 CM group compared to the MCF-12A CM group. A non-significant difference was observed in the rate of wound closure of the MCF-7 CM group compared to the MCF-12A CM group. Vertical bars denote mean ± SEM. Asterisks (*) denotes $p<0.05$. Representative scratch images can be found in **Figure 3.5.2**. Abbreviations: HUVECs, Human umbilical vein endothelial cells.

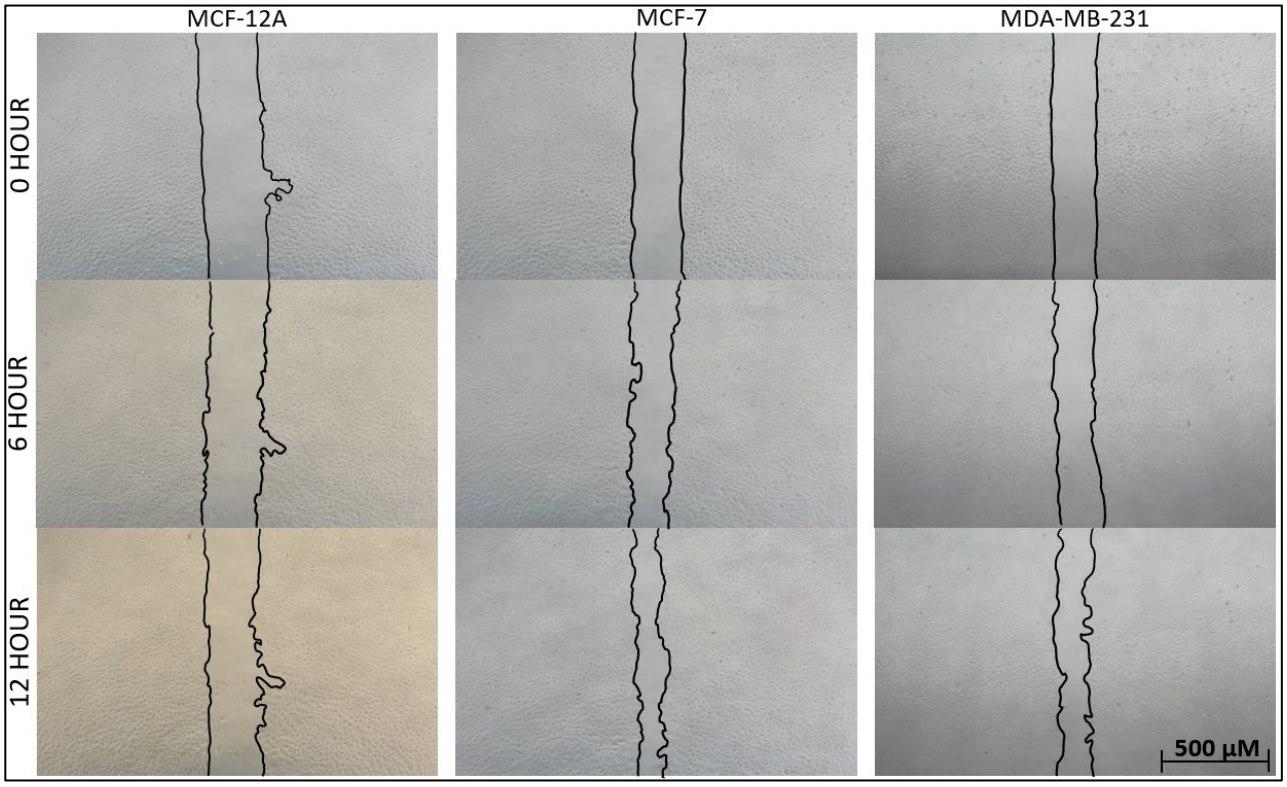

Figure 3.5.2: Representative phase contrast images of the scratch assay to assess cell migration of HUVECs. A scratch was made to the cell monolayer, cells were treated with conditioned media and mitomycin C was administered. Cells were subsequently imaged over a 24-hour period. Brightfield images were acquired with a Zeiss Olympus® CKX31 (Olympus®, GMBH Japan) inverted microscope equipped with an Axiocam 208 color camera and Zeiss Laboscope software (Carl Zeiss, Germany) at 4x (500 µM) magnification. Abbreviations: HUVECs, Human umbilical vein endothelial cells.

3.2.2 Tube Formation

A key aspect of a tube formation assay is the use of a basement membrane, which mimics the natural environment of endothelial cells, stimulating their inherent blood vessel forming behaviour in the presence of angiogenic inducers. This assay provides insight into cell migration, adhesion, protease activity and tube formation that lead to the formation of capillary-like structures in a meshed network with lumens (Carpentier et al., 2020; Arnaoutova & Kleinman, 2010).

The tube formation assay was used to assess the influence of breast cancer on the angiogenic activities of HUVECs. Cells were seeded on a basement membrane and treated at time point 0. The cells were subsequently imaged over a 24-hour period with an incubation monitoring system. The images taken over time provide the opportunity to observe the process as it happens with the use of a time-lapse. Tube formation can also be quantified with the use of software that analyzes factors such as the mesh network, tubule formation (cell elongation), and node formation (Carpentier et al.,

2020). However, for the purpose of this study, HUVEC tube formation in response to breast cancer was only subjected to qualitative assessment (**Figure 3.6**).

The mesh network of HUVECs treated with malignant CM, is more established (defined) at the 12-hour time point compared to the non-malignant CM group. The mesh network of the malignant CM groups is also more extensive at the 24-hour time point. Additionally, the lumens formed at the 24-hour time point of the malignant CM groups are bigger and more irregular. The nodes, which are the junctions with 3 or more cells, are less dense in the malignant CM groups at the 24-hour time point. Furthermore, the malignant CM groups possess more tube-like structures that exhibit a greater degree of elongation at the 24-hour time point.

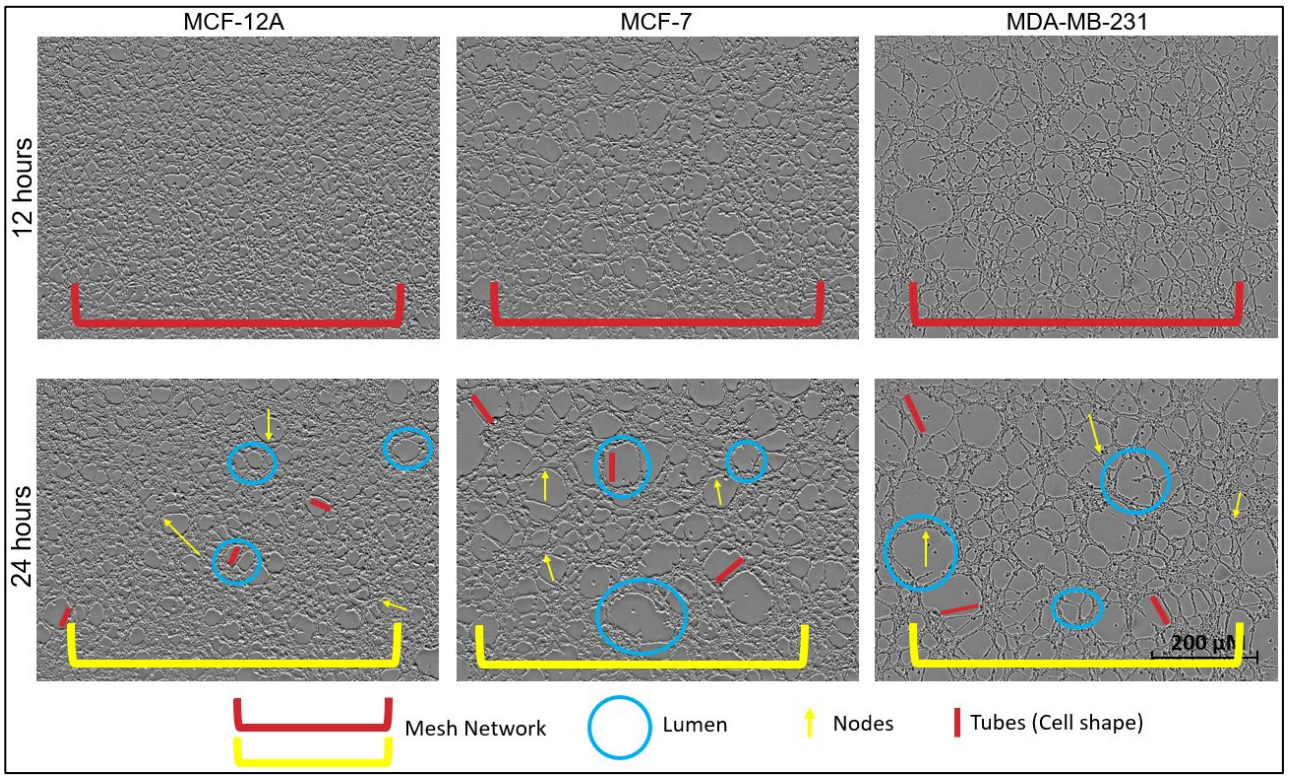

Figure 3.6: Cell monitoring to assess tube formation of HUVECs following conditioned media treatment (n=1). Conditioned medium (CM) was harvested from starved non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells. HUVECs were seeded on a basement membrane, treated with 100% CM and imaged over a 24-hour period with the Olympus Provi CM20 incubation monitoring system (Olympus Life Science, Japan). Abbreviations: HUVECs, Human umbilical vein endothelial cells.

Chapter 4: Discussion

In breast cancer, uncontrolled growth most commonly occurs in the mammary epithelial tissue, which is highly responsive to both local and systemic signals (Feng et al., 2018; Lukong, 2017). Cancer cells transform their anti-tumourigenic niche into a pro-tumourigenic niche, termed a TME by inducing a tumour or tumour-associated phenotype in stromal cells. Conditions that arise in the microenvironment such as hypoxia and inflammation further perpetuate the pro-tumourigenic nature of the TME (Son et al., 2017; Ziyad & Iruela-Arispe, 2011; Wiseman & Werb, 2002). Signalling from the stroma has been implicated in breast cancer progression and breast cancer cells have been observed to transform stromal cells, such as fibroblasts and immune cells, to acquire pro-tumourigenic phenotypes (Ingthorsson et al., 2010). Endothelial cells are not only recruited to provide an angiogenic response in response to nutrient and oxygen deprivation but are also transformed into TECs, subsequently acquiring pro-tumourigenic properties (Dudley, 2012). For example, TECs in breast cancer have been proven to promote cancer cell survival, stemness, invasion and metastasis (Ghiabi et al., 2014).

4.1 A tumour endothelial cell (TEC) phenotype in breast cancer compared to non-malignant expression profiles

The TEC phenotype is distinct from that of a normal endothelial cell – which provides insight into the functioning of the TME, insight into the mechanisms of action contributing to the disease aetiology and makes them more attractive targets for anti-angiogenic therapies (Hida, Hida & Shindoh, 2008). The first aims of the study were therefore to establish and characterize a TEC phenotype in breast cancer in response to paracrine breast cancer cell signalling and to compare it to changes induced by non-malignant paracrine signalling. Starvation, or nutrient deprivation, provided an appropriate model, because along with metabolic adaptions, cells are prompted to adopt adaptive responses, including angiogenesis (Püschel et al., 2020).

4.1.1 Cell viability

A WST-1 assay was used to assess the effect of breast cell CM on the cell viability of HUVECs as an assessment of HUVEC proliferation (**Figure 3.1**). To the best of our knowledge, a change in cell viability of endothelial cells in response to non-malignant breast epithelial cell starvation is a novel finding. Compared to the control, the cell viability of all the CM groups significantly increased. The release of stimulatory signals that may be responsible for the increase in cell viability can be

attributed to the fact that cells trigger adaptive responses that include targeting endothelial cells and stimulating angiogenesis in response to starvation (Püschel et al., 2020; Longchamp et al., 2018). This is in agreement with another study where the authors reported that extracellular vesicles (EVs) isolated from starved MCF-7 and MDA-MB-231 CM increased the cell viability of HUVECs (Sun, Zhang, et al., 2022).

Significant differences in cell viability were also noted between the non-malignant CM group and both malignant CM groups, and between the malignant MCF-7 and MDA-MB-231 CM groups. This agrees with the findings of Akiyama et al. (2012), who found that skin cancer cell CM stimulated endothelial cell (adult phenotype) proliferation. Tu et al. (2009) also observed increased cell viability in HUVECs exposed to CM from lung cancer cells exposed to 24-hour serum starvation and subsequent 24-hour low serum conditions (5% FBS). Additionally, differences in non-malignant versus malignant responses may be attributed to the overexpression of factors in malignant cells. A key example is that of Dhami et al. (2022), who found that breast cancer cells, MCF-7s and MDA-MB-231 specifically, release and stimulate the release of factors, such as VEGF and angiopoietins, which stimulate endothelial cells under low-serum conditions compared to non-malignant cells (Dhami et al., 2022). The release of these factors may therefore translate to and be exacerbated by starvation. These findings are further supported by the pro-angiogenic and pro-survival profiles of HUVECs in co-culture with lung cancer cells (Cheng et al., 2017).

The differential response of signalling from MCF-7 and MDA-MB-231 cells may be attributed to the fact that these cells respond differently to starvation (preliminary data not shown). Nakhjavani et al. (2017) showed that MCF-7s adapt to harsh environmental conditions, such as starvation, by modulating mitochondrial activity. MDA-MB-231s, on the other hand, possess a survival advantage during serum starvation conferred by the high levels of mutant p53 (Hui et al., 2006). Gómez et al. (2016) reported a greater release of VEGF in MCF7 CM, compared to MDA-MB-231 in response to starvation.

4.1.2 Cell morphology

It is well established that morphological changes are key in establishing a vascular network (Tsuji-Tamura & Ogawa, 2018). Phase contrast imaging was used to assess the effect of non-malignant and malignant breast cell CM on the cell morphology of HUVECs (**Figure 3.2**). Qualitative analysis revealed cell elongation, lumen formation (hollow spaces) and a form of branching, which occurred to a greater extent in HUVECs treated with CM from malignant cells, with more branching in the MDA-MB-231 CM group compared to the MCF-7 CM group.

This agrees with the findings of Gómez et al. (2016), who used a similar model with HUVECs, MCF-7, MDA-MB-231 and ZR-75-30 cells, where they observed a change in cell morphology for all CM groups but that the degree of cell elongation occurred to a greater extent in the invasive MDA-MB-231 and ZR-75-350 CM groups. Their findings were corroborated by actin cytoskeletal reorganization and stress fibre formation. However, they did not observe any changes in response to CM from a non-malignant breast cell line (MCF-10A) (Gómez et al., 2016). These findings are also supported by the research of Dhami and co-workers who observed that MDA-MB-231 cells elicited a greater response from HUVECs compared to MCF-7 cells in low serum conditions (Dhami et al., 2022). HUVECs in co-culture with lung cancer cells also exhibited altered cell morphology (Cheng et al., 2017). Furthermore, blood vessels of breast tumour tissues were also reported to be abnormal and dilated, while influenced by breast cancer phenotype (Senchukova et al., 2015).

4.1.3 TEC marker gene expression

TECs are thought to have a distinct genetic profile compared to normal endothelial cells (Nagl et al., 2020; Dudley, 2012). The relative gene expression of VEGFR2, MDR1, Biglycan and LOX, which are reported to be upregulated in TECs, were analyzed with RT-qPCR.

4.1.3.1 VEGFR2

VEGFR2 is the major pro-angiogenic receptor that is pro-tumourigenic through its angiogenic and angiogenesis-independent activity (Lian et al., 2019; Zarkada et al., 2015; Guo et al., 2010). Its main pro-tumourigenic functions in endothelial cells include cell proliferation, survival and migration (Matsuda et al., 2010; Smith et al., 2010; Bussolati et al., 2003).

VEGFR2 expression significantly decreased in all CM groups compared to the control (**Figure 3.3.1**). To the best of our knowledge, the expression of VEGFR2 in response to starvation in non-malignant cells is a novel finding. Contradictory to our findings, Bussolati et al. (2003) report constant upregulation of VEGFR2 in renal cancer TECs and labelled it as a marker of endothelial cell proliferation. Akiyama et al. (2012) observed VEGFR2 upregulation in adult endothelial cells exposed to skin cancer CM. Matsuda et al., (2010), similarly reported upregulated VEGFR2 in isolated TECs from oral, skin and renal tumours compared to resting normal endothelial cells (mouse model). Additionally, VEGFR2 was also observed to be upregulated in TECs isolated from high-metastatic skin tumours compared to low-metastatic tumours (mouse model) (Ohga et al., 2012). However, Zhang et al. (2010) reported that VEGF-dependent angiogenesis involves upregulated VEGFR2 and downregulated VEGFR1 in normal tissues and downregulated VEGFR2 and upregulated VEGFR1 in tumour tissues.

A decrease in the relative gene expression of VEGFR2 was unexpected as cells secrete VEGF to activate VEGFR2 to activate angiogenesis, which is normally overexpressed in cancer cells (Guo et al., 2010). The CM of MCF-7 and MDA-MB-231 cells was evaluated by Gómez et al. (2016) who found that VEGF was especially high in the MCF-7 CM. The decreased VEGFR2 expression as observed in our results does also not correlate with the increased cell viability, as well as the upregulation of MDR1, which is upregulated downstream of VEGF/VEGFR2 activity (Akiyama et al., 2012). Considering that proliferation pathways are rapid, it is possible that the expression of VEGFR2 after 24 hours may not be representative of its activity. The analysis of relative VEGFR2 expression may therefore require time-dependent analysis and may serve as an indicator of rapid transcription.

4.1.3.2 MDR1

MDR1 is a pro-tumourigenic membrane efflux pump that provides acquired cross-drug resistance (Akiyama et al., 2012). MDR1 expression significantly increased in the MCF-12A CM group compared to the control and MCF-7 and MDA-MB-231 CM groups (**Figure 3.3.2**). Akiyama et al. (2012) reported that CM harvested from normal skin cells did not upregulate MDR1 mRNA, but they do not show the data. They do however report that there were no significant differences between the CM of normal skin cells compared to the CM of adult endothelial cells, while the concentration of VEGF in the cancer cell CM treatment group was significantly upregulated. They did not mention whether the cells were exposed to serum starvation but based on their study methodology and their VEGF findings, it can be assumed that the cells were not serum starved. Therefore, to the best of our knowledge, MDR1 has not been investigated in the context of a physiological response to starvation.

Studies have mainly been done in the blood brain barrier, where the endothelial cells constitutively express MDR1 (Cordon-Cardo et al., 1990). While adult endothelial cells generally lack MDR1 expression, HUVECs express MDR1 mRNA. MDR1 is upregulated under hypoxic conditions as it possesses a hypoxia responsive element, conferring drug resistance in the absence of previous exposure to drugs in adult endothelial cells (Comerford, Cummins & Taylor, 2004; Comerford et al., 2002). Yet, considering that similar responses would be elicited in non-malignant and malignant cells in response to starvation, it may be possible that MDR1 is upregulated in the non-malignant CM group for a different reason. In glioblastoma's MDR1 is considered as a sign of vessel co-option – or vessel hijacking (Annese et al., 2022). No studies have reported that MDR1 plays a role in physiological angiogenesis, however, there are two factors that should be considered. Firstly, MDR1 has a wide array of endogenous MDR1 substrates, including reactive oxygen species, cytokines, phospholipids, and steroids (Chen, Sun, et al., 2020; Rockwell, 2004; Hoffmeyer et al., 2000). In TECs, MDR1 is upregulated downstream of angiogenic and stress pathways (Cheng et al., 2017;

Akiyama et al., 2012; Comerford, Cummins & Taylor, 2004; Sang et al., 2003; Wang et al., 2003). Our findings may, therefore, provide potential insight of MDR1 upregulation during physiological angiogenesis and while its expression in cancer is uncontrolled, it may be tightly regulated in non-malignant settings.

Contradictory to our findings in breast cancer CM groups, Akiyama et al. (2012) reported MDR1 upregulation in endothelial cells (adult phenotype) from day 1 of their 5-day skin cancer CM treatment period. Huang et al. (2013) similarly found MDR1 expression in both HUVECs and adult endothelial cells in response to doxorubicin treatment. Taken together, these findings thus emphasize that in TECs, MDR1 expression is acquired through physiological expression, exposure to hypoxia and exposure to cytotoxic agents (Wang et al., 2018). It does, however, not indicate that the HUVECs exposed to breast cancer CM do not have a drug resistant phenotype, as there are several markers that may provide similar effects, including multidrug resistance associated protein and breast cancer resistance protein (Mehta & Siddik, 2009).

4.1.3.3 Biglycan

Biglycan is a proteoglycan that is pro-angiogenic, pro-inflammatory and undetectable under normal conditions (Berendsen et al., 2014; Calabrese et al., 2011; Hu et al., 2016; Nastase, Young & Schaefer, 2012; Obika et al., 2013). No significant differences were observed in Biglycan expression between any of the CM groups (**Figure 3.3.3**).

Contradictory to our findings, Yamamoto et al. (2012) identified Biglycan as a TEC marker using TECs isolated from skin cancer and normal skin endothelial cells (mouse model) and associated it with autocrine angiogenic stimulation. *In vivo* results revealed expression in tumour blood vessels, with little to no expression in normal vessels. They also detected Biglycan in the serum of cancer patients compared to healthy controls. Similarly, Maishi et al. (2016) reported upregulation in TECs and identified DNA methylation as the mechanism of action.

Our findings also do not agree with the cell morphology and cell migration results, as Biglycan has been implicated as a key role player in altered cell morphology and cell migration (Maishi et al., 2016; Yamamoto et al., 2012). It may, however, be that this also requires time dependent analysis – as Biglycan mRNA expression in HUVECs has been observed to peak at 3 hours and significantly decrease after 12 hours (Obika et al., 2013).

4.1.3.4 LOX

LOX is a tightly regulated oxidase that when overexpressed, negatively affects endothelial health and becomes pro-tumourigenic and pro-angiogenic (Adamopoulos et al., 2016; Saatci et al., 2020; Sun, Ma et al., 2022; Zhu et al., 2021). Downstream consequences of LOX overexpression include cell migration, tube formation, and altered cell morphology and metastasis (Osawa et al., 2013). We have observed a significant increase in LOX expression in the MCF-12A CM group compared to control and the MCF-7 CM group (**Figure 3.3.4**). To the best of our knowledge, the expression of LOX in a non-malignant response to starvation is a novel finding.

Our search has revealed that studies have only analysed LOX under non-angiogenic physiological and pathological conditions (Laczko & Csiszar, 2020; Baker et al., 2013; Lucero, Mäki & Kagan, 2011; Laczko et al., 2007). LOX is upregulated during of hypoxia, and interacts with various angiogenic role players, including the PDGF-B receptor, Akt and VEGF – contributing to both blood vessel formation and remodelling (Laczko & Csiszar, 2020; Saatci et al., 2020). LOX upregulation in the non-malignant CM group therefore provides insight into the role that LOX may play during physiological angiogenesis.

The lack of LOX upregulation in the breast cancer CM groups does not support our cell morphology and cell migration results. It also contradicts the findings of Osawa et al. (2013), who reported higher expression in TECs and tumour blood vessels, as compared to normal endothelial cells and normal vessels, respectively, using a mouse model. Concomitantly, Baker et al. (2013) established that LOX is a driving force in tumour angiogenesis through the stimulation of endothelial cells. They found that LOX stimulates angiogenesis in breast and colorectal cancer (mouse models). Furthermore, LOX derived from lung cancer cell spheroids has shown to be key to angiogenesis in HUVECs upstream of VEGF activity (Yang et al., 2019). The latter provides a possible explanation that cancer cells serve as the initial source of LOX and that microenvironmental interactions are key contributors to its upregulation.

4.1.4 TEC marker protein expression

TECs are thought to express markers specific to their tumourigenic phenotype compared to normal endothelial cells. The protein expression of TEM7, TEM8 and CXCR7, which are reported to be TEC specific markers, were analyzed with Western blots. Western blots were also used to assess the protein expression of MCM2, which a marker of cell proliferation.

4.1.4.1 TEM7

TEM7 is a cell surface protein that is pro-angiogenic and pro-tumourigenic. Its pro-tumourigenic roles include inflammation, invasion and metastatic dissemination (Geng et al., 2021; Pietrzyk & Wdowiak, 2019; Zhang et al., 2015; Fuchs et al., 2007). Its specific tumourigenic functions in endothelial cells are unknown but it is associated with pathological angiogenesis (Yamaji et al., 2008). No significant differences in TEM7 expression were observed between any of the CM groups (**Figure 3.4.1**).

Our results differ with the findings of St. Croix et al. (2000) and their classification of TEM7 as a TEC specific marker. Nanda, Buckhaults et al., (2004) subsequently confirmed TEM7 upregulation in various tumour types, including colon and brain tumours using mouse and human tissues. TEC-specific expression was also confirmed in brain cancers (human model) with an experimental and bioinformatic approach (Beaty et al., 2007). Yet, it is in support of the association of TEM7 with circulating endothelial cells (progenitor and adult) and their involvement in angiogenesis (tumour infiltrating blood vessels), as opposed to transformed stromal endothelial cells (Mehran et al., 2014).

4.1.4.2 TEM8

TEM8 is a cell surface receptor that is pro-angiogenic and pro-tumourigenic. Its pro-tumourigenic functions include tumour growth, angiogenesis and metastasis (Xu et al., 2021; Høye et al., 2018; Cao et al., 2016; Opoku-Darko et al., 2011; Rmali et al., 2004). However, its specific functions in TECs are unknown. As depicted in **Figure 3.4.2**, a significant increase in TEM8 expression is observed in the MCF-12A and MCF-7 CM groups compared to the control. A significant difference is also observed between the MCF-12A and MDA-MB-231 CM groups.

The expression of TEM8 in a non-malignant response to starvation has not been observed before, to the best of our knowledge, and, therefore, our finding that TEM8 is upregulated in the MCF-12A CM group contradicts with the classification of TEM8 as a TEC specific marker (St. Croix et al., 2000). The difference in TEM8 expression in the MCF-12A CM group compared to the MDA-MB-231 CM group contradicts the upregulation of TEM8 in tumour blood vessels, while the latter agrees with the upregulation in the MCF-7 CM group (Davies et al., 2004; St. Croix et al., 2000). Opoku-Darko et al. (2007) found that cancer cells increased both the membrane bound and soluble TEM8 in HUVECs in co-culture with breast cancer cells (HS578T cell line).

An essential difference in our study is that we analysed soluble TEM8 while most studies analyzed membrane bound TEM8 (Ding et al., 2021; Høye et al., 2018; Opoku-Darko et al., 2011; Davies et al., 2004). Xu et al. (2021) reported TEM8 overexpression in TNBC cells and not stromal cells and classified it as marker of TNBC cell vascular mimicry (human model). Davies et al. (2004) also

classified TEM8 as a more tumour specific marker but also detected TEM8 in normal breast tissues, albeit at a significantly lower degree compared to breast cancer tissues (human models). Our supplementary data confirms the expression of TEM8 in breast cancer cells and possibly non-malignant breast cells – depending on which band or whether both 60 kDa bands are variants of membrane bound TEM8, as displayed in **Supplementary Figure S3**. The soluble receptor, however, is exclusively expressed in HUVECs.

As previously mentioned, studies reporting on TEM8 expression have only used normal unstimulated endothelial cells and physiological angiogenesis in wound healing and in the corpus luteum, which are not good comparisons for breast tissue angiogenesis. Our findings highlight a potential role for soluble TEM8 in adult physiological angiogenesis, and not only embryonic angiogenesis, however, further investigation is required. Verma et al. (2011) reported that TEM8 modulates blood vessel density and patterning in developmental angiogenesis using chicken chorioallantoic membranes. Our results also support a role for TEM8 to contribute to the TEC phenotype in breast cancer.

4.1.4.3 CXCR7

CXCR7 is a membrane receptor associated with endothelial activation and transformation to a pro-tumourigenic phenotype. It is associated with pro-tumourigenic activities such as tumour growth, angiogenesis, and metastasis (Li et al., 2020; Liu et al., 2020; Qian et al., 2018; Miao et al., 2007). It disrupts endothelial cell homeostasis and is associated with vascular dysfunction, being implicated in cell proliferation, altered cell morphology, cell migration, and enhanced invasive properties, amongst others (Totonchy et al., 2013, 2014).

Maishi et al. (2012) labelled CXCR7 as a novel TEC marker for renal cell carcinoma. They confirmed the upregulation of CXCR7 in: TECs isolated from renal, skin and oral cancers compared to normal skin endothelial cells in a mouse model; in TECs isolated from renal cancers in a human model; and in normal endothelial cells following treatment with cancer cell CM. Additionally, Würth et al. (2011) reported CXCR7 expression in several types of cancers, including colon and lung cancers; and they also reported the lack thereof in normal endothelial cells. There appears to be consensus that CXCR7 expression and upregulation is limited to tumour vessels and not normal vessels (Maishi et al., 2012; Monnier et al., 2012; Würth et al., 2011).

The increased protein level (albeit not significant) is in agreement with our cell viability and cell morphology findings of our non-malignant CM group. These changes are attributed to CXCR7 functioning in endothelial cells observed in transfection studies (Totonchy et al., 2013, 2014). However, the lack of change in our malignant CM groups does not agree with the changes in proliferation, migration, and tube formation. In TECs, CXCR7 has specifically been implicated in

resistance to serum starvation, migration, and tube formation (Yamada et al., 2015). Additionally, VEGF signalling is also implicated in CXCR7 upregulation (Qian et al., 2018; Yamada et al., 2015; Costello et al., 2008).

4.1.4.4 MCM2

Mini-chromosome maintence-2 (MCM2) is part of a family of proteins involved in DNA replication and their activity serves as an indication of active cell proliferation (Nowińska & Dzięgiel, 2010). MCM2 was therefore used to assess the influence of breast cell CM on the proliferative activity of HUVECs; however, no significant differences were observed between any of the treatment groups (**Figure 3.4.4**).

These results do not support our cell viability results and studies that reported an increase in cell proliferation of HUVECs exposed to cancer cells. For example, Sun, Zhang et al. (2022) concluded that MCF-7 and MDA-MB-231-derived EVs enhanced cell proliferation of HUVECs using cell viability assays, clone formation assays and western blot analysis of the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) proliferation pathway. Lung cancer cells have also been observed to stimulate cell viability and the PI3K/Akt pathway, which is associated with cell proliferation and survival, in HUVECS (Cheng et al., 2017; Tu et al., 2009). Additionally, skin cancer cells were observed to enhance the proliferation and resistance to serum starvation of adult endothelial cells where the PI3K/Akt pathway was also activated (Akiyama et al., 2012).

4.1.5 Summary of TEC phenotype in breast cancer

A concerted effort has been made to highlight the differences between normal endothelial cells and TECs as these cells are key to cancer progression and serve as potential anti-angiogenic and anti-tumourigenic targets. TECs have been characterized to differ in their capacity to proliferate, cell appearance and the expression of markers. Studies classify an array of markers to be specific to TECs but as previously mentioned, their shortfall has been comparing it to endothelial cells that are unstimulated and/or from functionally different tissues. Here we report that endothelial cells are stimulated to a greater degree by breast cancer signalling – supporting the transformation of endothelial cells to TECs. We also report that paracrine signalling from starved breast cancer cells alone is not sufficient to elicit the upregulation of markers that are key to the TEC phenotype, with the exception of TEM8. The lack of upregulated gene expression of markers reported to be elevated in TECs may be attributed to the role that other cells and microenvironment constituents play. Yet, paracrine signalling from starved non-malignant cells was sufficient to elicit the upregulation of these

markers – indicating that in the breast tissue, these markers are not specific to a malignant phenotype but also play a role in physiological angiogenesis (**Figure 4.1**).

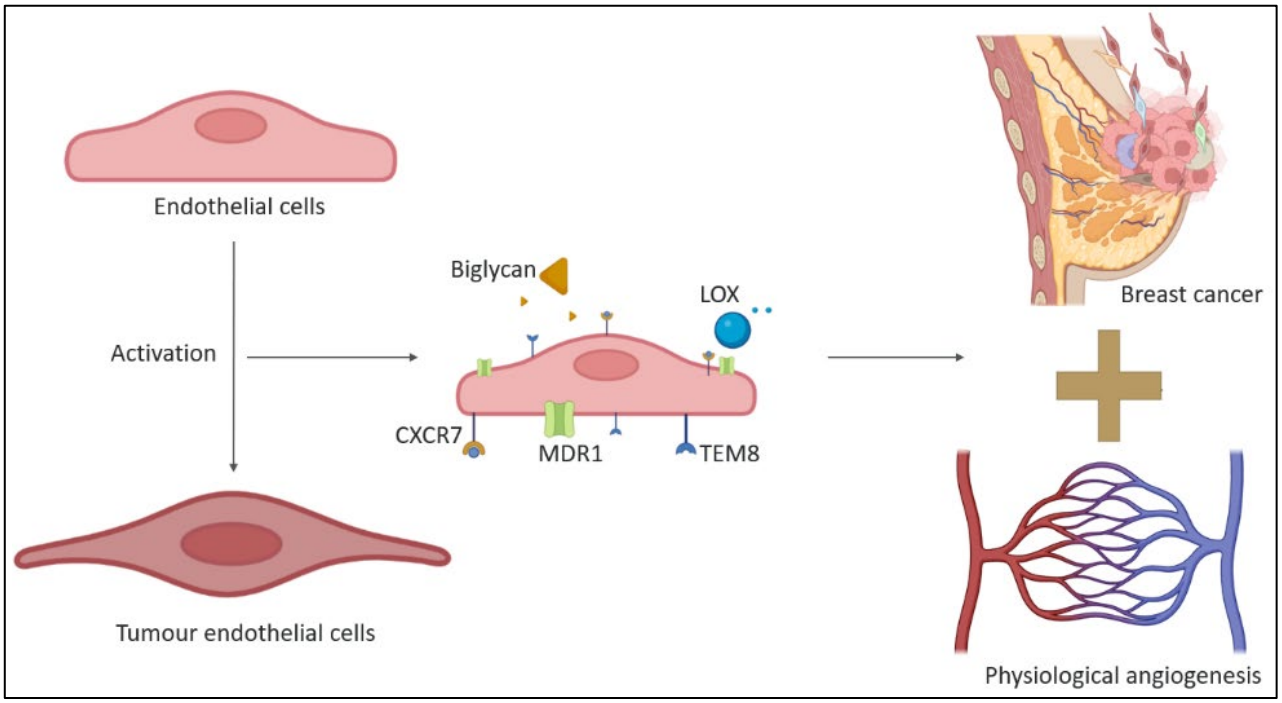

Figure 4.1: A summary of a tumour endothelial cell phenotype in breast cancer. Breast cancer cells do transform endothelial cells to tumour endothelial cells. However, the markers that are thought to be specific to a malignant phenotype are also playing a role in physiological angiogenesis (Created in Biorender.com). Abbreviations: CXCR7, Chemokine CXC receptor 7; LOX, Lysyl oxidase; MDR1, Multidrug resistance 1; TEM8, Tumour endothelial marker 8.

4.2 The influence of breast cancer on angiogenesis

Tumour angiogenesis is a central component of cancer progression (Hanahan & Weinberg, 2011). It provides oxygen and nutrients to the growing tumour, and as the gatekeepers to the circulatory system, facilitate metastasis. The absence of angiogenesis thus stunts cancer progression (Hida, Hida & Shindoh, 2008; Kerbel & Folkman, 2002; Kumar et al., 2001).

Paracrine signalling is a powerful mediator of angiogenesis through the release of pro-angiogenic factors such as VEGF, bFGF and IL-8 by cancer cells (Cheng et al., 2017; Kerbel & Folkman, 2002). Cancer cells and the surrounding tumourigenic cells are therefore able to enhance the angiogenic ability and activity of endothelial cells, the degree of which is determined by the balance of pro- and anti-angiogenic factors (Buchanan et al., 2012; Somanath et al., 2006).

Angiogenesis has been investigated in many studies in the context of breast cancer, however, all cancer-related angiogenic studies make comparisons to resting 'normal' endothelial cells, as opposed to activated or stimulated endothelial cells. The second aim of the study was therefore to investigate the difference between physiological and tumour angiogenic processes.

4.2.1 Cell migration

Endothelial cells migrate to invade the tissue towards angiogenic signals, which guide the newly forming sprouts. In the TME, a plethora of pro-angiogenic factors are released and the process often overstimulated (Lopes-Bastos, Jiang & Cai, 2016; Bergers & Benjamin, 2003). A scratch assay was employed to assess the effect of breast cancer CM on cell migration compared to non-malignant breast CM in response to starvation. The MCF-7 CM group elicited a significant increase in the percentage wound closure of HUVECs compared to the MCF-12A CM group (**Figure 3.5.1**).

The study by Sun, Zhang et al. (2022) revealed that EVs derived from MCF-7 and MDA-MB-231 CM increased the migratory activity of HUVECs using a scratch assay and western blot analysis of the JAK2/STAT3 pathway, which is also in agreement with some of our results – with the exception of the MDA-MB-231 CM group. Tu et al. (2009) also found that HUVECs exposed to lung cancer CM (24-hour serum starvation and subsequent 24-hour low serum conditions), exhibited increased migratory activity. HUVECs in co-culture with lung cells also exhibited higher migratory activity when compared to HUVECs cultured alone (Cheng et al., 2017). Yet in contrast to our findings that MCF-7 CM has a greater impact on cell migration, Ohga et al. (2012) observed that TECs isolated from highly metastatic tumours were more migratory compared to TECs isolated from low-metastatic tumours (mouse model, skin cancer). Their findings lead to the conclusion that highly metastatic tumour derived TECs have a more pro-angiogenic phenotype. Interestingly, Liang et al. (2018) implicated Angiopoietin 1 as the key factor secreted by MCF-7s that influences HUVEC migration.

4.2.2 Tube formation

Blood vessels in tumours are disorganized, unevenly distributed and follow tortuous paths. They are hyperpermeable with defective endothelial junctions and layers (Hida, Hida & Shindoh, 2008; Mcdonald & Choyke, 2003; Hashizume et al., 2000). A tube formation assay was used to assess the effect of breast cell CM on the tube-forming ability of HUVECs and several qualitative differences were noted (**Figure 3.6**). The mesh network (interconnected branching network) of HUVECs treated with malignant CM, was more established (defined) at the 12-hour time point compared to the MCF-12A CM group. The mesh network of the malignant CM groups was also more extensive at the 24-

hour time point. Additionally, the lumens formed at the 24-hour time point of the malignant CM groups were bigger and more irregular. The nodes, which are the junctions with 3 or more cells, were less dense in the malignant CM groups at the 24-hour time point. Furthermore, the malignant CM groups possessed more tube-like structures that exhibited a greater degree of elongation at the 24-hour time point. We do, however, interpret these differences as hyperactivation (overstimulation) of angiogenesis in response to signalling from breast cancer CM as compared to non-malignant breast CM.

This is in agreement with Marchetti et al. (2008), who reported an increase in the length of tubes of HUVECs treated with CM from starved MCF-7 and MDA-MB-231 cell lines compared to unstimulated ECs. While their control group did not form functional tubes, their CM groups, and especially the MCF-7 CM group, fully formed tubes. EVs derived from MCF-7 and MDA-MB-231 CM also enhanced HUVEC tube formation by increasing the number of tubules formed (Sun, Zhang et al., 2022). Additionally, Matsuda et al. (2010) confirmed the tube forming ability of TECs isolated from mouth, kidney and skin tumours. Cheng et al. (2017) subjected HUVECs in co-culture with lung cancer cells to a tube formation assay. The qualitative differences observed compared to unstimulated HUVECs were densely formed tubes and not nodes compared to our findings. Quantitatively, the cells in co-culture presented with significantly higher numbers of nodes and tubes. Cells in co-culture were also more resistant to apoptosis. Furthermore, Zhou et al. (2008) reported an increased angiogenic ability in immortalized TECs compared to normal endothelial cells.

4.2.3 Summary of angiogenesis in breast cancer

While tumour angiogenesis, or neoangiogenesis, has been widely studied in response to hypoxia, several reports also indicated that nutrient restriction evoked the same angiogenic responses through the release of proangiogenic factors (Püschel et al., 2020). Here we report that, compared to physiological angiogenic signalling from non-malignant breast epithelial cells, the angiogenic response induced by breast cancer cell signalling is distinct and hyperactivated. The degree of hyperactivation is, however, influenced by the breast cancer phenotype. Although *ex vivo* results report that TECs from high metastatic tumours are more pro-angiogenic, our results indicate that in response to starvation, low metastatic tumours elicit a greater angiogenic response through paracrine signalling. Differences *in vivo* may be attributed to the TME of high metastatic tumours that possess a greater array of surrounding tumourigenic cells compared to low metastatic tumours, while *in vitro* results may be attributed to the difference in response to starvation of cancer cells (Hui et al., 2006; Li et al., 2021).

Chapter 5: Conclusion

It is evident that breast cancer cells release paracrine signals to communicate with surrounding cells. They release a plethora of stimulatory and pro-tumourigenic factors that recruit and alter cells to establish a TME. Processes such as angiogenesis, are also stimulated to such a degree that it is abnormal compared to the physiological responses. The aims of this study were to evaluate a TEC phenotype in breast cancer and assess the paracrine influence of breast cancer on angiogenesis – and compare the responses to a non-malignant response. The results of this study confirmed that breast cells signal endothelial cells in response to starvation and that the signalling from breast cancer cells is more extensive. Pertaining to a TEC phenotype in breast cancer, the endothelial cells did present with altered phenotypes but paracrine signalling in response to starvation was not sufficient to induce the expression of most of the characteristic markers. These markers were also shown to not be specific to TECs and were also upregulated in response to non-malignant paracrine signalling. Pertaining to angiogenesis in breast cancer, breast cancer cells hyperactivated angiogenic processes compared to physiological angiogenic signalling. We, therefore, agree that stromal endothelial cells are an important source of TECs, but that the degree of change induced does depend on the breast cancer phenotype. Taking the above findings into consideration, we conclude that in breast cancer, endothelial cells and the angiogenic processes are altered and therefore key to cancer progression but that a TEC specific phenotype remains to be defined (**Figure 4.2**).

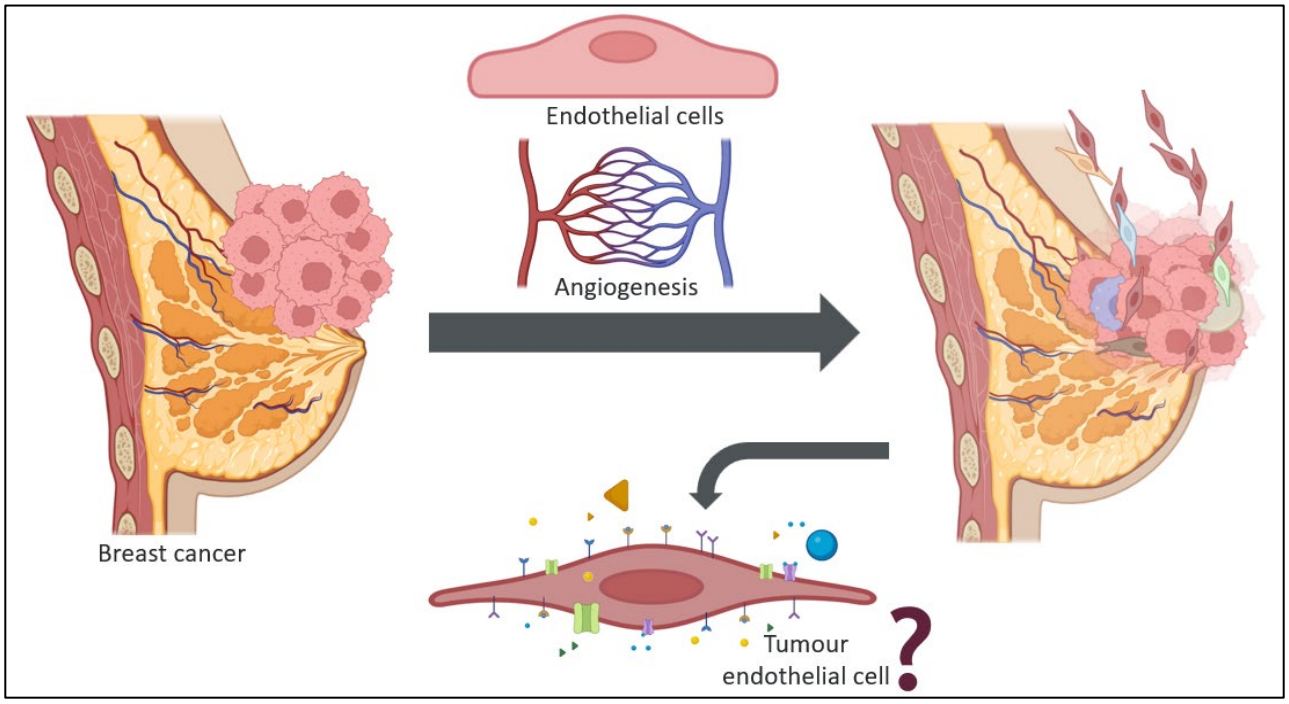

Figure 4.2: Breast cancer cells alter endothelial cells and the angiogenic response but a tumour endothelial cell specific phenotype in breast cancer remains to be defined. (Created in Biorender.com).

Chapter 6: Future directions and limitations

A limitation of this study was the use of HUVECs and their embryonic gene expression profile. These findings should therefore be confirmed with adult endothelial cells and in *in vivo* mouse models.

Ideally, endothelial cells in co-culture with breast cells and specifically breast tumour spheroids would have provided a better indication of a TEC phenotype in breast cancer. Supplementary data did indicate an increase in cell viability of HUVECs in co-culture with MCF-12A and MDA-MB-231 cells (**Supplementary Figure S4**).

Inducing hypoxia in cancer cells would have provided another angle of mimicking the TME. The use of cobalt chloride proved to be unsuccessful in both MCF-7 and MDA-MB-231 cell lines (**Supplementary data-S1**). Additional methods of hypoxia induction should be tested. Another way to mimic TME interactions would be to expose cancer cells to CM from normal cells, before harvesting CM.

The effect of breast cancer on endothelial cell function (dysfunction) using a co-culture model would have provided insight into why breast cancer patients present with cardiovascular complications down the line.

The protocol for the western blot detection of TEM8 should be further optimized.

The protein expression of VEGFR2, MDR1, Biglycan and LOX; and the gene expression of TEM7, TEM8 and CXCR7 should also be evaluated to obtain a complete picture of the activity of the markers.

The relative gene expression of markers, especially VEGFR2 and Biglycan, should be assessed in response to a range of treatment periods – for example: 2, 4, 6 and 12 hours.

Due to time restrictions, we were unable to quantify our tube formation assay results. The tube formation assay should also be assessed and quantified at different densities as preliminary data (not shown) indicated clear differences.

The use of aortic ring assays in an *ex vivo* mouse models would have modelled the angiogenic response within one assay.

Cell cycle analysis and mitochondrial assays would provide insight into why HUVECs exhibited an increase in cell viability.

The effect of doxorubicin on the breast cancer cell-endothelial cell interactions should also be investigated.

Once these changes are made and the experiments are repeated and confirmed in *in vivo* models, it would provide insight into targeting TECs in adjuvant breast cancer therapies, while mitigating the consequences of traditional anti-angiogenic approaches.

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Supplementary Information

1. Mimicking the tumour microenvironment (TME): establishing hypoxia in an *in vitro* breast cancer model

Hypoxia is a well-established characteristic of cancer that affects cancer progression and treatment response (Walsh et al., 2014). It influences the progression of the TME and plays a key role in the transformation to a TEC phenotype (Comşa et al., 2015; Nejad et al., 2021). We, therefore, aimed to investigate the contribution of hypoxia to the TEC phenotype.

Obtaining and managing low O2 incubators and environments is difficult and extremely expensive, especially compared to the use of a chemical approach. Therefore, to induce hypoxia, Cobalt Chloride ($CoCl_2$) was utilized, which mimics hypoxia through the stabilization of the HIF-1α subunits. This model is well established in the investigation of hypoxia and provides an affordable, easy, and consistent model for hypoxia induction (Muñoz-Sánchez & Chánez-Cárdenas, 2019; Wu & Yotnda, 2011).

To establish a hypoxia in a breast cancer model, the breast cancer cell lines and $CoCl_2$ were utilized. Cells were treated with different concentrations of $CoCl_2$ obtained from literature. Initially the MDA-MB-231 was utilized, then the MDA-MB-231 and the MCF-7 cell lines were used. Cell viability, the gene expression of HIF-1α, and the gene and protein expression of VEGF were subsequently assessed and used to confirm whether the cells triggered a hypoxic response. The protein expression of HIF-1α was excluded due to a non-functioning antibody.

1.1 Methods

1.1.1 Induction of hypoxia: Cobalt Chloride ($CoCl_2$) preparation and treatment approach

$CoCl_2$ is a hypoxic mimetic agent which functions through the stabilization of hypoxia-inducible factor-alpha subunits. Its use for hypoxia induction is well-established and provides an affordable, easy, and consistent model for hypoxia induction (Muñoz-Sánchez & Chánez-Cárdenas, 2019; Wu & Yotnda, 2011). $CoCl_2$ Hexahydrate was purchased from Sigma-Aldrich® (Cat # C8661) and prepared according to the manufacturer's recommendation. Briefly, $CoCl_2$ was dissolved in distilled H_2O (dH_2O) to achieve a concentration of 25 mM, filter-sterilized and stored at $4^{O}C$. Before use, it was diluted in cell culture medium according to the desired concentrations. MDA-MB-231 cells were treated with 10 µM, 25 µM and 100 µM $CoCl_2$ for 24 hours (**Figure S1.1-A**). MDA-MB-231 cells were

treated with 10 µM and 25 µM $CoCl_2$ and MCF-7 cells with 100 µM and 150 µM $CoCl_2$ for 48 hours (**Figure S1.1-B**).

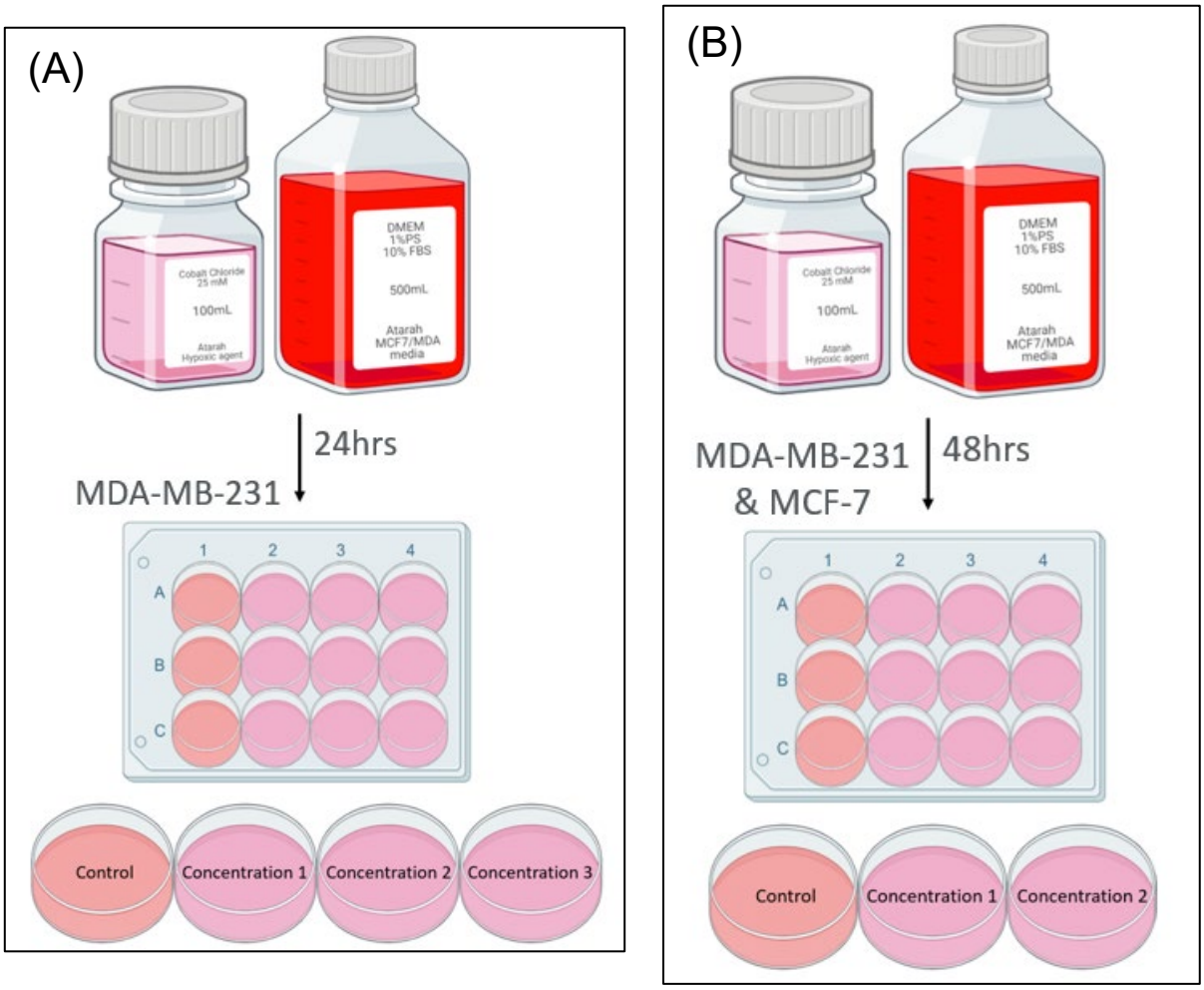


Figure S1.1: Treatment approaches used to treat (A) MDA-MB-231 cells for 24 hours and (B) MDA-MB-231 and MCF-7 cells for 48 hours. (Created in Biorender). Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal Bovine Serum.

1.1.2 RT-qPCR: Primer design

Amplification of the genes of interest was achieved with the following primers designed by Dr Tanja Davis: **HIF-1α** (NM_181054.3) forward: 5'-GCAGCAACGACACAGAAACT-3', reverse: 5'-TTCAGCGGTGGGTAATGGAG-3' (amplicon length, 131 bp); and **VEGF** (NM_001025366.3) forward: 5'-CTTCAAGCCATCCTGTGTGC-3', reverse: 5'-TGTGCTGTAGGAAGCTCATCTC-3' (amplicon length, 161 bp). Primers were designed to span exon-exon boundaries. The reference genes, **HPRT1** and **RPLP0**, were used to normalize quantitation cycle values of **HIF-1α** and **VEGF**.

1.2 Hypoxia induction following 24-hour Cobalt Chloride ($CoCl_2$) Treatment

MDA-MB-231 cells were treated with 10 µM, 25 µM and 50 µM $CoCl_2$ for 24 hours using complete culture medium. The WST-1 assay was employed to assess whether $CoCl_2$ promotes proliferation or cell death in MDA-MB-231 breast cancer cells. As depicted in **Figure S1.2-A**. No significant differences were observed between any of the treatment groups ($p>0.05$).

During hypoxia, the HIF-1α subunit is stabilized and dimerizes with the HIF-1β subunit, forming the HIF1 transcription factor that activates genes possessing hypoxia responsive elements (Huang et al., 1998; Wang et al., 1995; Semenza et al., 1991). VEGF is a hypoxia-inducible gene activated to trigger angiogenic, proliferative and survival pathways (Hashimoto & Shibasaki, 2015; Ferrara & Kerbel, 2005; Iyer et al., 1998). RT-qPCR was employed to assess the gene expression of HIF-1α and VEGF. As depicted in **Figure S1.2-B**, $CoCl_2$ induced a dose-dependent decrease in the HIF-1α gene expression. No significant differences were, however, observed in VEGF gene expression (**Figure S1.2-C**). Western blots were used to assess the protein expression of VEGF. Similar to its gene expression, no significant differences were observed in VEGF protein expression (**Figure S1.2-D**).

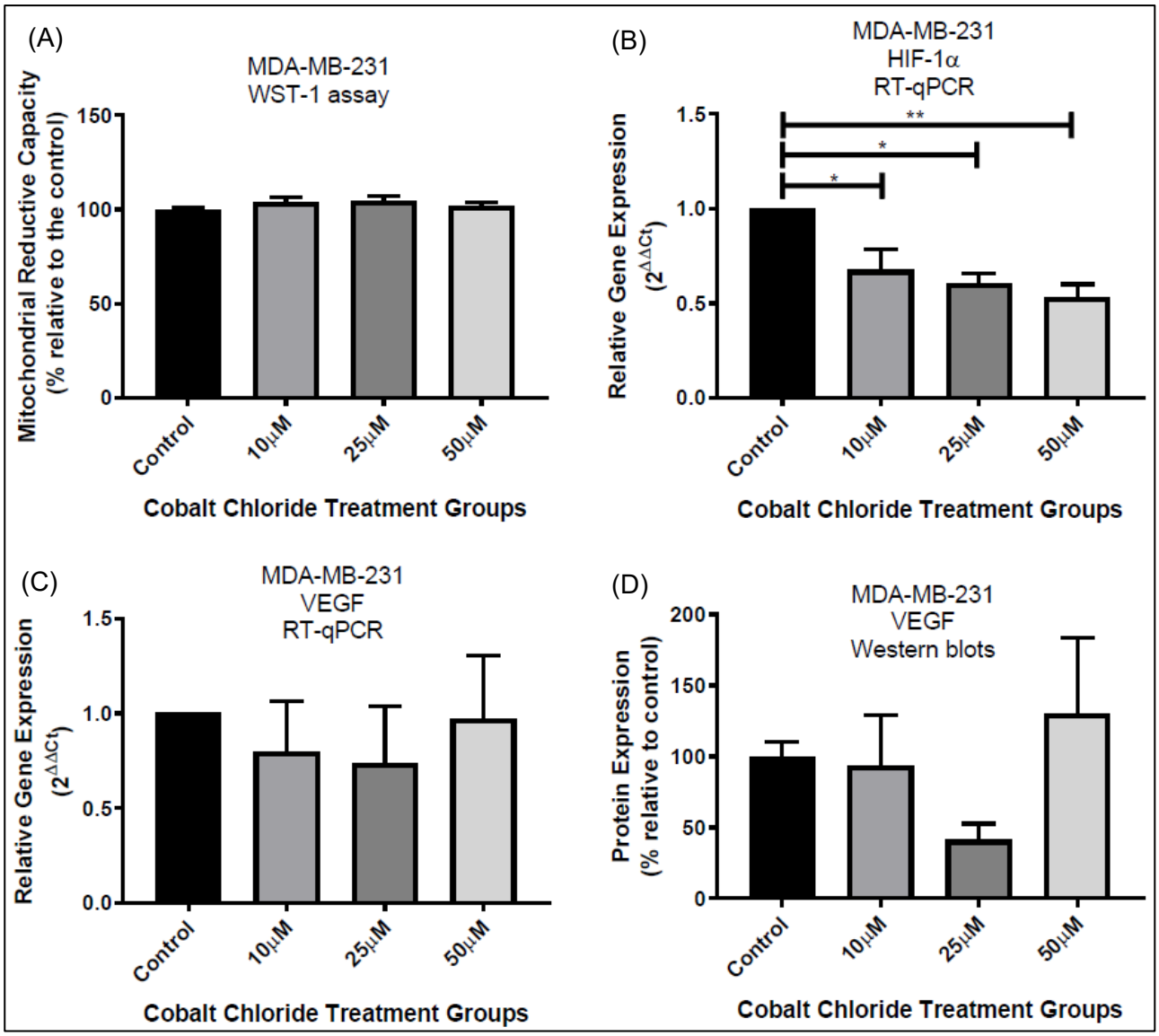


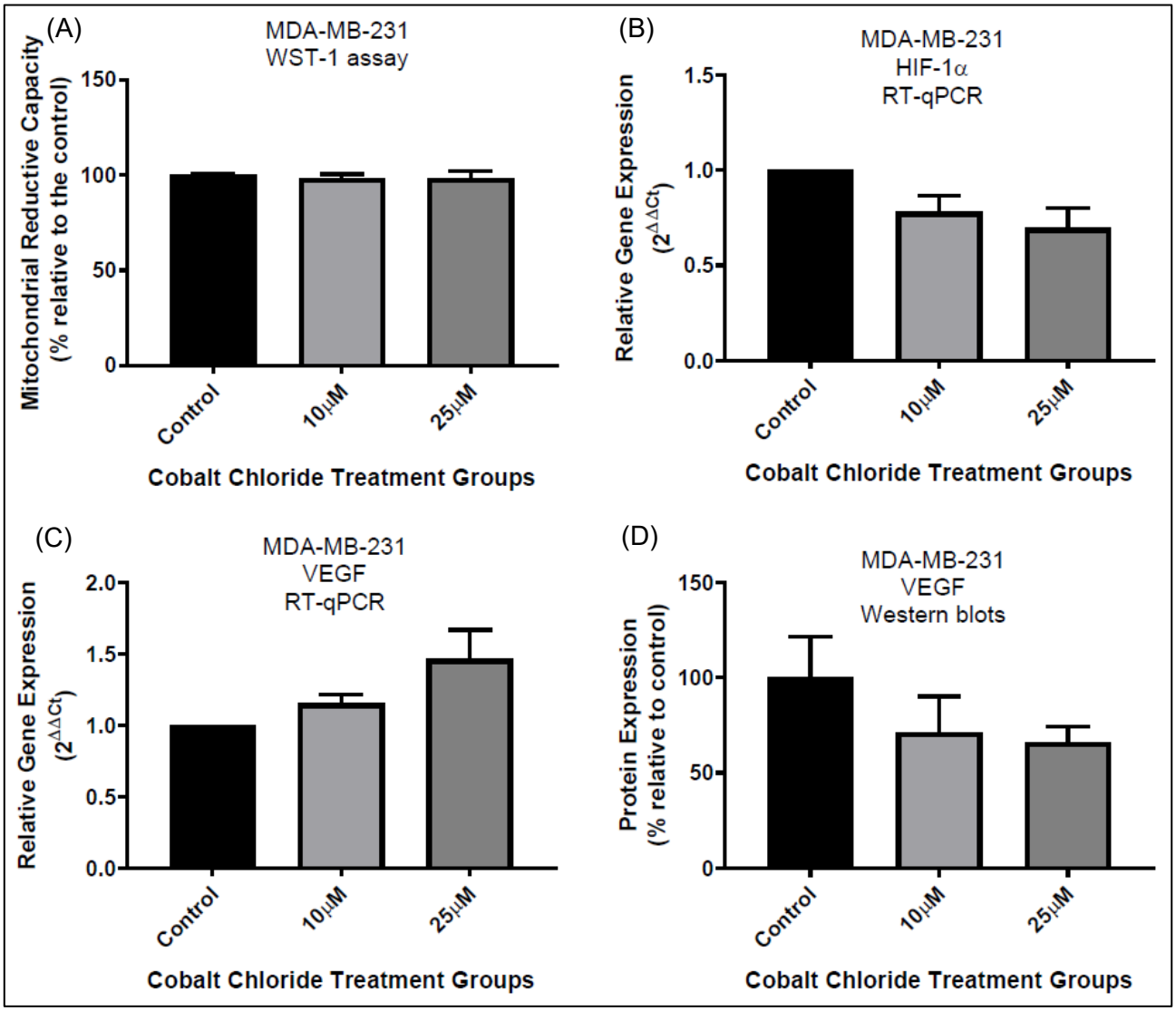
Figure S1.2: Hypoxia induction in the MDA-MB-231 cell line following 24-hour $CoCl_2$ treatment (n=3). (A) **Mitochondrial reductive capacity assessed using a WST-1 assay as an indicator of cell viability.** No significant differences were observed ($p>0.05$). Results are expressed as mean ± SEM. (B) **RT-qPCR analysis of HIF-1α involved in hypoxic regulation.** $CoCl_2$ induced a dose-dependent decrease in HIF-1α expression. Vertical bars denote mean ± SEM. Asterisks (*, **) denotes $p<0.05$ & $p<0.01$, respectively. (C) **RT-qPCR analysis of VEGF involved in hypoxic regulation.** No significant differences were observed ($p>0.05$). Vertical bars denote mean ± SEM. (D) **Western blot analysis to detect VEGF expression**. No significant differences were observed ($p>0.05$). Results are presented as mean ± SEM. Abbreviations: $CoCl_2$, Cobalt Chloride; HIF, Hypoxia inducible factor; RT-qPCR, Reverse Transcriptase-quantitative polymerase chain reaction; VEGF, Vascular endothelial growth factor; WST-1, Water tetrazolium salt-1.

1.3 Hypoxia induction following 48-hour Cobalt Chloride ($CoCl_2$) Treatment

Optimization of our hypoxia model lead the inclusion of two breast cancer cell lines and a longer treatment period.

1.3.1 MDA-MB-231 cell line

MDA-MB-231 cells were treated with 10 µM and 25 µM $CoCl_2$ for 48 hours using complete culture medium. The WST-1 assay was employed to assess whether $CoCl_2$ promotes proliferation or cell death in MDA-MB-231 breast cancer cells. As depicted in **Figure S1.3-A**. No significant differences were observed between any of the treatment groups ($p>0.05$). RT-qPCR was employed to assess the gene expression of HIF-1α and VEGF. As depicted in **Figure S1.3-B** and **Figure S1.3-C**, no significant differences were observed in HIF-1α and VEGF gene expression, respectively ($p>0.05$). Western blots were used to assess the protein expression of VEGF. Similar to its gene expression, no significant differences were observed in VEGF protein expression ($p>0.05$) (**Figure S1.3-D**).



FigureS1.3: Hypoxia induction in the MDA-MB-231 cell line following 48-hour $CoCl_2$ treatment (n=3). (A) **Mitochondrial reductive capacity assessed using a WST-1 assay as an indicator of cell viability.** No significant differences were observed ($p>0.05$). Results are expressed as mean ± SEM. (B) **RT-qPCR analysis of HIF-1α involved in hypoxic regulation.** No significant differences were observed ($p>0.05$). Vertical bars denote mean ± SEM. (C) **RT-qPCR analysis of VEGF involved in hypoxic regulation.** No significant differences were observed ($p>0.05$). Vertical bars denote mean ± SEM. (D) **Western blot analysis to detect VEGF expression**. No significant differences were observed ($p>0.05$). Results are presented as mean ± SEM. Abbreviations: $CoCl_2$, Cobalt Chloride; HIF, Hypoxia inducible factor; RT-qPCR, Reverse Transcriptase-quantitative polymerase chain reaction; VEGF, Vascular endothelial growth factor; WST-1, Water tetrazolium salt-1.

1.3.1 MCF-7

MCF-7 cells were treated with 100 μM and 150 μM $CoCl_2$ for 48-hours using complete culture medium. The WST-1 assay was employed to assess whether $CoCl_2$ promotes proliferation or cell

death in MCF-7 breast cancer cells. As depicted in **Figure S1.4-A**. cell viability decreased in the 100 µM $CoCl_2$ group ($p<0.05$). RT-qPCR was employed to assess the gene expression of HIF-1α and VEGF. HIF-1α gene expression significantly decreased in the 150 µM $CoCl_2$ group ($p<0.05$) (**Figure S1.4-B)**. However, no significant differences were observed in VEGF gene expression ($p>0.05$) (**Figure S1.3-C**). Western blots were used to assess the protein expression of VEGF. Similar to its gene expression, no significant differences were observed in VEGF protein expression ($p>0.05$) (**Figure S1.3-D**).

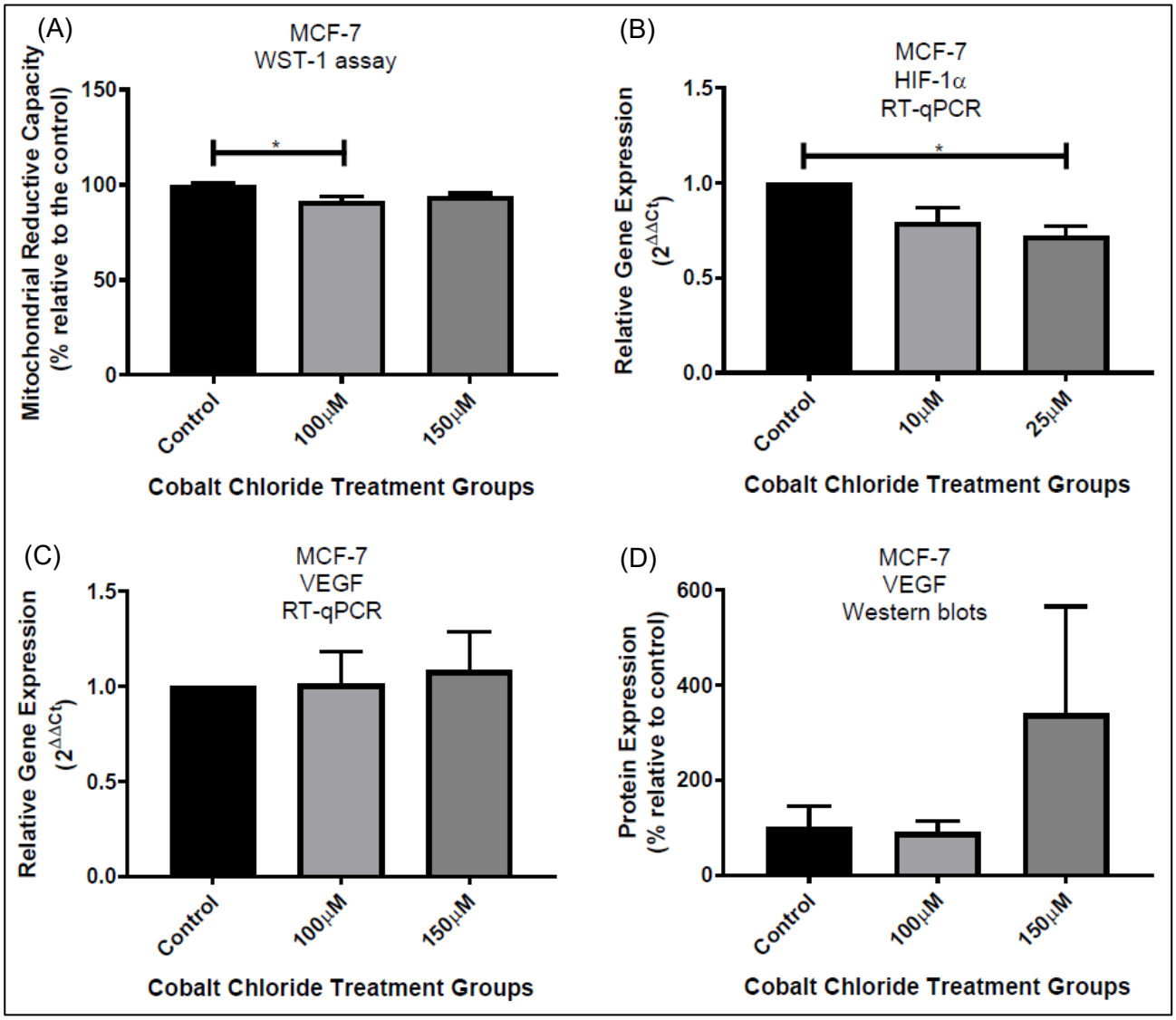


Figure S1.4: Hypoxia induction in the MCF-7 cell line following 48-hour $CoCl_2$ treatment (n=3). (A) **Mitochondrial reductive capacity assessed using a WST-1 assay as an indicator of cell viability.** No significant differences were observed ($p>0.05$). Results are expressed as mean ± SEM. (B) **RT-qPCR analysis of HIF-1α involved in hypoxic regulation.** No significant differences were observed ($p>0.05$). Vertical bars denote mean ± SEM. (C) **RT-qPCR of VEGF involved in hypoxic regulation.** No significant differences were observed ($p>0.05$). Vertical bars denote mean ± SEM. (D) **Western blot analysis to detect VEGF expression**. No significant differences were observed ($p>0.05$). Results are presented as mean ± SEM. Abbreviations: $CoCl_2$, Cobalt Chloride; HIF, Hypoxia inducible factor; RT-qPCR, Reverse Transcriptase-quantitative polymerase chain reaction; VEGF, Vascular endothelial growth factor; WST-1, Water tetrazolium salt-1.

1.3.3 Conclusion

We are limited in our capacity to comment on HIF-1α expression, but the decrease in HIF-1α gene expression may be attributed to the rapidity of the hypoxic pathway. However, the importance of VEGF in the hypoxic pathway and crosstalk with endothelial cells, coupled with the lack of VEGF upregulation, suggests that the cells have not activated or have not sufficiently activated the hypoxic pathway. We therefore conclude that we were unsuccessful in inducing hypoxia and due to a lack of time, we are unable to further optimize this model.

2. The effect of supplemented conditioned medium (CM) on endothelial cells.

For CM dose response optimization, HUVECs were treated with 50% and 100% of CM of the different cell lines. An MTT was used to assess mitochondrial reductive capacity as an indirect assessment of cell viability and proliferation. As depicted in **Figure S2.1**, all CM groups elicited a significant increase in cell viability compared to the control. In the malignant CM groups, the 50% CM group elicited a greater response compared to the respective 100% CM groups. The question arose of whether the supplements remaining in the CM were responsible for the enhanced effect. The effect of the cell culture medium of the breast cells on the HUVECs was subsequently assessed with a MTT assay. As shown in **Figure S2.2**, the cell culture medium of both the non-malignant and malignant cell lines significantly increased the cell viability of HUVECs.

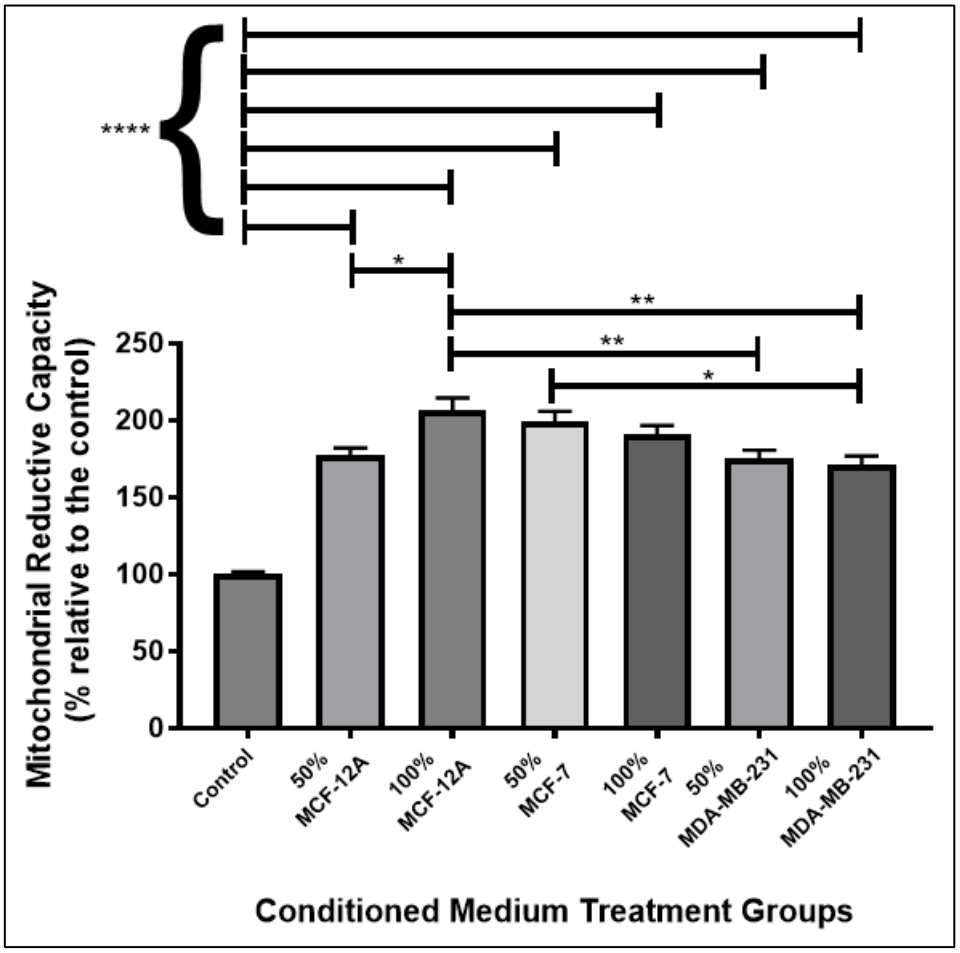


Figure S2.1: Mitochondrial reductive capacity as an indirect measure of cell viability following dose response conditioned media treatments. Conditioned medium (CM) was harvested from non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells treated with supplemented culture medium. HUVECs were treated with 50% and 100% CM for 24 hours and subjected to a MTT assay. Significant differences were observed between all CM groups. Results are expressed as mean ± SEM. Asterisks (*, **, ****) denotes $p<0.05$, $p<0.01$ and $p<0,0001$, respectively. Abbreviations: HUVECs, Human umbilical vein endothelial cells; MTT, 3-(4,5-dimethyllthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole.

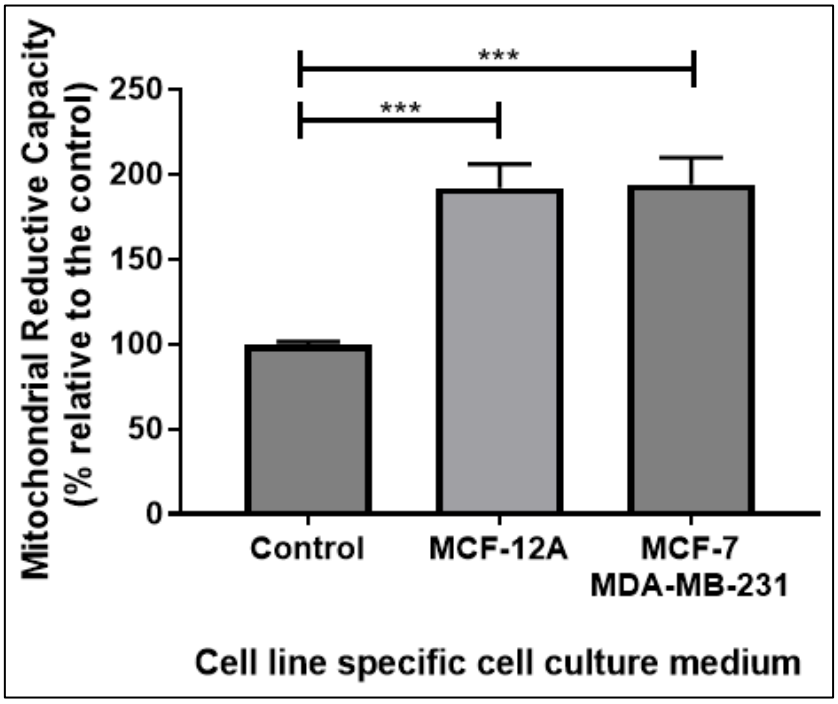


Figure S2.1: Mitochondrial reductive capacity as an indirect measure of cell viability following dose response conditioned media treatments. Conditioned medium (CM) was harvested from non-malignant (MCF-12A) and malignant (MCF-7 & MDA-MB-231) breast cells treated with supplemented culture medium. HUVECs were treated with 50% and 100% for 24 hours and subjected to a MTT assay. Significant differences were observed between all CM groups. Results are expressed as mean ± SEM. Asterisks (*, **, ****) denotes $p<0.05$, $p<0.01$ and $p<0,0001$, respectively. Abbreviations: HUVECs, Human umbilical vein endothelial cells; MTT, 3-(4,5-dimethyllthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole.

3. TEM8 Optimization

TEM8 is expressed as five splice variants that encode membrane bound and soluble receptors. The designation of the different bands was based on the results of Hotchkiss et al. (2005).

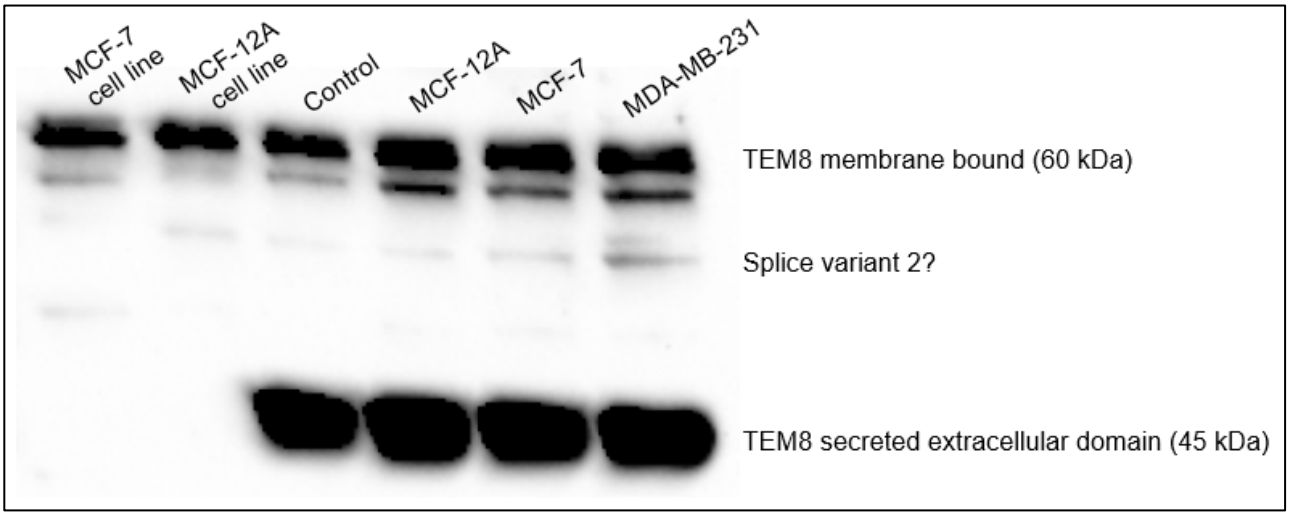


Figure S3: Representative detection of Western blot detection of TEM8 variants. Abbreviations: TEM8, Tumour endothelial marker 8.

4. The effect of co-culture interactions on the cell viability of endothelial cells

HUVECs were co-cultured with non-malignant (MCF-12A) and malignant (MDA-MB-231) cells for 24 hours and subsequently subjected to a MTT assay (**Figure S4**). A MTT assay was used to assess mitochondrial reductive capacity as an indirect assessment of cell viability and proliferation.

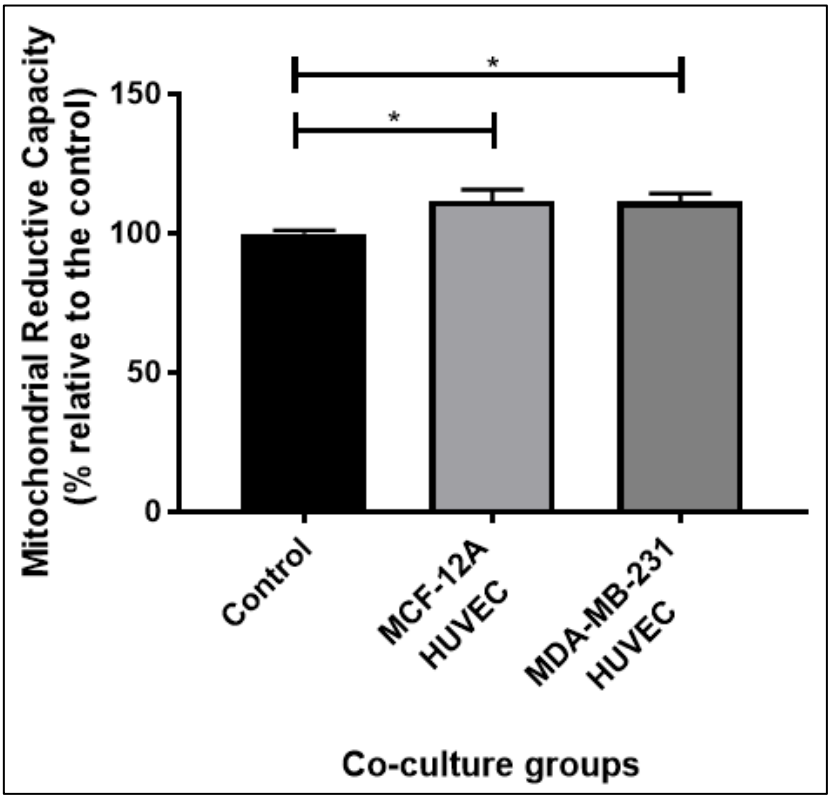


Figure S4**: Mitochondrial reductive capacity as an indirect measure of cell viability following co-culture of HUVECs and breast cells.** HUVECs were co-cultured with non-malignant (MCF-12A) and malignant (MDA-MB-231) breast cells. After 24 hours, HUVECs subjected to a MTT assay. Significant differences were observed in both co-culture groups compared to the control. Results are expressed as mean ± SEM. Asterisks (*) denotes $p<0.05$. Abbreviations: HUVECs, Human umbilical vein endothelial cells; MTT, 3-(4,5-dimethyllthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole.

Appendixes

Appendix A: General reagents

PBS (2 L)

1. Dissolve the following chemicals in 1.8 L dH_2O:
   - 16.001 g NaCl
   - 0.400 g KCl
   - 2.882 g Na_2HPO_4
   - 0.481 g KH_2PO_4
2. Adjust to pH 7.4
3. Fill to 2 L
4. Autoclave
5. Store between 4°C and RT

Appendix B: RT-qPCR protocols

Gels: RNA & PCR

Gel recipes		
Application	%	Quantities
Checking RNA • Also shows if DNA contamination • V_f = 6 µL • 80 V	1%	1 g Agarose in 100 mL 1x TAE
Checking primer binding • PCR/Post-qPCR • 90 V, 1h-1h15m • V_f = 12 µL	2%	2 g Agarose in 100 mL 1x TAE
	3%	3 g Agarose in 100 mL 1x TAE
Calculations		
RNA gels: RNA (1g/1uL), H2O & loading dye (5x -> 1x) = 6 µL PCR/Post-qPCR gels: 10 µL PCR product + 2 µL loading dye = 12 µL		

1. Assemble gel kit & check if level

2. Weigh Agarose
3. Add to 100 mL TAE in Erlenmeyer flask
4. Boil in microwave until dissolved – swirl in between
5. Allow to cool down – until the flask can be held
6. Add pre-stain (5 µL in 100 mL)
7. Pour gel into the mold
8. Check for bubbles and dirt, and move to the side with pipette (can affect migration)
9. Insert desired comb (the less wells, the better)
10. Allow to set until it's no longer transparent
11. When ready, load according to the step you're carrying out
12. Run black to red
13. Image on Chemidoc

RNA Extraction

1. Remove growth media
2. Add Trizol reagent directly to cells
    - 350 µL per well in a 12-well plate.
3. Pipet up & down several times to homogenize, and incubate for 5 minutes
    - Rinse over plate as well, solution should become less "slurry"
    - Samples can be stored at 4°C O/N or at -20°C for up to one year
4. Transfer samples to microcentrifuge tubes
5. Add 0.2 mL chloroform per 1 mL Trizol used, and incubate for 2-3 minutes
    - Invert tube thoroughly several times (may also require light shaking) - chloroform must be mixed well into sample to prevent phenol contamination of aqueous phase
    - For example: 210 µL per group if using a 12-well plate
6. Centrifuge samples for 15 minutes at 12 000 g (12 200 rpm) at 4°C
    - Samples will separate into three phases: lower red phenol-chloroform phase, middle white interphase (may be "thready", and stick to side of eppie), and an upper colourless aqueous phase
    - Handle samples carefully when removing from centrifuge to prevent mixing of phases
    - Spinning at is crucial 4°C in this step
7. Transfer the aqueous phase, containing the RNA, into a new tube
    - Angle the tube at 45° and slowly pipet out the solution while keeping the tip just below the meniscus

- Do not attempt to collect the entire aqueous phase – DNA and phenol-chloroform contamination can have significant consequences in downstream applications

8. Add 0.5 mL Isopropanol per 1 mL Trizol and incubate for 10 minutes
    - Lightly shaking/ or inverting is CRUCIAL.
    - For example: 525 µL per group is using a 12-well plate
9. Centrifuge for 10 minutes at 12 000 g (12 200 rpm) at 4°C or RT
    - When placing the tubes in the centrifuge, take note of which side the pellet will form – if no pellet is visible, continue as normal but avoid touching the area that should contain the pellet
    - Total RNA precipitates as a white gel-like pellet at the bottom of the tube
10. Resuspend the pellet in 1 mL 75% EtOH per 1 mL Trizol used, and vortex sample briefly
    - RNA can be stored in 75% EtOH for one week at 4°C or one year at -20°C
    - For example: 1050 µL per group is using a 12-well plate
11. Centrifuge for 5 minutes at 7500 g (9500 rpm) at 4°C or RT
    - When removing supernatant, be careful to not pull up the pellet – the RNA pellet, when in 75% EtOH, does not stick very tightly to the eppie
12. Repeat wash step if required
    - For instance, if aqueous phase had a pink colour
13. Air dry the pellet for 5-10 minutes
    - Remove as much as possible of the supernatant
    - Place samples under light to speed up the process
    - After 5 minutes, monitor pellets carefully. Do not let the RNA pellet dry-out to ensure total solubilisation of RNA. Partially dissolved RNA samples have an A260/280 ratio < 1.6. Dried-out RNA pellets will become colourless.
14. Resuspend the RNA pellet in 25-50 µL RNase-free H_2O by pipetting
15. Incubate samples at 55-60°C for 10-15 minutes
    - 57°C for 12 minutes works fine
16. Determine yield with Nanodrop
    - Blank with H_2O
    - Dilutions may be required, Nanodrop has an upper limit of 1200 ng/µL
    - Note the A260/280 ratio - pure high-quality RNA will have a ratio close to 2.0, but anything between 1.8 and 2.0 is fine
17. Store RNA in aliquots at -80°C

Removing genomic DNA

- Use icepack in DSG -20°C – stored upside down to avoid ice crystals
- DNase + DNase buffer
- EDTA – afterwards to stop the reaction – RNA hydrolyzes during heating with divalent cations in the absence of a chelating agent
- Do calculation for 1 µg RNA:

$$\frac{Average\ [RNA]}{1\ \mu l} = \frac{1000\ ng\ RNA}{Volume\ needed}$$

1. Add to an RNase-free tube:

RNA	-80°C	$\frac{600\ ng\ RNA}{6.6\ \mu l} = \frac{1000\ ng\ RNA}{X}$ Can use 750 ng RNA/7.5 μl
10x reaction buffer with MgCl2	-20°C	
DNase I, RNase-free (#EN0521)		
DEPC-treated water (#R0601)		

   → Make a master mix of buffer and enzyme

   → Add smallest volume last

2. Incubate at 37°C for 30 min
3. Add __ µL 50 mM EDTA and incubate at 65°C for 10 min

   → 1:1 ratio to DNAse I

4. Use the prepared RNA as a template for reverse transcription

   → Use DNA-free RNA for all steps from this point onwards

   → Can store these samples at -20°C

Synthesize cDNA

- LunaScript™ RT SuperMix Kit
- After DNA removal
- Always include a no RT control to ensure no DNA contamination
- Need 500 ng RNA for the reaction

$$\frac{600\ ng\ RNA}{6.6\ \mu l} = \frac{500\ ng\ RNA}{X}$$

$$X = 5.5\ \mu l$$

Protocol as determined by reverse transcriptase reagent used:

1. Prepare cDNA synthesis reaction mix
2. *Prepare one no-RT control – choose random samples per cDNA 'set'*
    - *Do from time to time*
3. Prepare no-template control
4. Perform listed incubation steps in PCR machine
5. Store at -20°C

qPCR

- Prepare excel calculation sheet
- According to Luna® Universal qPCR master mix protocol

1. Dilute cDNA
2. Make qPCR master mixes per primer/concentration
3. Load cDNA (smallest volume first)
4. Load master mix
5. Remember NTC or NRTs
6. Seal properly with special film without touching the inside

Appendix C: Western blot reagents

Modified RIPA buffer (100 mL)

1. Dissolve the following reagents in 50 mL dH_2O:
    - 790 mg Tris-base [± 65 mM]
    - 900 mg NaCl [± 154 mM]
2. Adjust to pH 7.4
3. Add:
    - 10 mL 10% NP-40 [1%]
    - 10 mL 10% Na-deoxycholate (prepared as 1 g Na-deoxycholate in 10 mL dH_2O) [1%]
4. Stir until clear
5. Add:
    - 5 mL of 100 mM EDTA (5 mM) (prepared as 1.46 g EDTA in 50 mL dH_2O, pH 8.0) [± 5 mM]

- 5 mL of 100mM EGTA (5mM) (prepared as 1.90 g EGTA in 50 mL dH_2O, pH 8.0) [± 5 mM]
- 1 mL of 10% SDS (prepared as 50 g SDS in 500 mL dH_2O) [0.1%]

6. Fill to 100 mL dH_2O
7. Aliquot into tubes
8. Store at 4°C.

Before use:

1. To 1 mL RIPA buffer, add:
   - 42 µL Protease inhibitors cocktail
   - 5 µL 200 mM Na3VO4
   - 5 µL 200 mM NaF
   - 5 µL 200 mM Phenylmethylsulfonyl Fluoride (PMSF) (add last)

10X TBS (2 L)

1. Add:
   - 48.4g Tris
   - 160g NaCl
1. Dissolve in 1.5 L dH_2O
2. Adjust to pH 7.6 with HCl
3. Fill to 2L
4. Store at RT

1X TBS-T (2 L)

1. Add & stir:
   - 200 mL TBS
   - 1800 mL
   - 2 mL Tween-20
2. Store at RT

Appendix D: Bradford Assay and Sample preparation

Bradford reagent stock solution (5x)

1. Dissolve 500 mg Coomassie Brilliant Blue G-250 in 250 mL 95% ethanol
2. Add 500 mL Phosphoric acid
3. Mix thoroughly
4. Make up to 1 L with dH_2O
5. Filter and store @ 4°C

Bradford reagent working solution (1x)

1. Dilute stock in 1:4 ratio with dH2O
2. Filter using 2 filter papers at the same time, until solution is a light brown colour
3. Store at in the dark at RT

Bradford assay

1. If needed, thaw protein samples on ice
2. Thaw a 2mg/mL BSA stock solution
3. Prepare BSA working solution (200µg/mL) by diluting 100ul BSA + 900ul
4. Vortex
5. Prepare Bradford standards as follows, in duplicate:

Table 7: Volumes of components used to establish Bradford standards.

µg Protein	**2 mg/mL BSA**	**dH_2O**	**Bradford Reagent**
0 (Blank)	0µl	100µl	900µl
2	10µl	90µl	900µl
4	20µl	80µl	900µl
8	40µl	60µl	900µl
12	60µl	40µl	900µl
16	80µl	20µl	900µl
20	100µl	0µl	900µl

6. Vortex protein samples

7. For each Bradford sample, add 5µl of protein sample to 95µl dH_20 and 900µl Bradford reagent. Prepare samples in duplicate.
8. Vortex Bradford samples and standards
9. Incubate samples for 10 minutes off ice
10. Zero spectrophotometer with blank and set @ 595 nm
11. Read absorbencies
12. Draw standard curve in Excel graph and plot values of sample to determine protein sample concentrations

Laemmli's sample buffer stock solution

i. Dissolve 9.09 g Tris in 100 mL dH_2O
ii. Add 6 mL 10% SDS
iii. Adjust to pH 6.8
iv. Fill to 150 mL dH_2O
v. To a glass beaker:

- Add 60 g Glycerol
- Add 99.9 mL of the above solution to the Glycerol
- Add 26.4 g SDS and dissolve thoroughly
- Add 0.225g Bromophenol Blue and dissolve thoroughly

vi. Fill to 225 mL with dH_2O
vii. Store in the dark at RT

Laemmli's sample buffer working solution

In a fumehood,

1. To a microcentrifuge tube, add 850 µL sample buffer
2. Add 150 µL β-mercaptoethanol
3. Vortex

Final concentrations when adding sample buffer to protein sample in ratio of 1:2

- 62.5 mM Tris, pH 6.8
- 4% SDS
- 10% Glycerol
- 0.03% Bromophenol Blue

- 5% β-mercaptoethanol

Sample preparation

1. Prepare a working solution of Laemmli's sample buffer
2. Prepare samples by adding the appropriate amount of protein sample to sample buffer (as determined with Bradford Assay)
3. In order to give equal amounts of protein for loading = 25 µg
4. Vortex samples
5. Store as ready-made samples @ -80°C

Appendix E: Western blot protocol

Day 1

1. Prepare BioRad Fast Cast Stain-Free gels or Self Cast according to instructions
   - Gels can be made beforehand and stored in the fridge in wetted paper towels and sealed in cling wrap
2. Thaw protein samples on ice
3. Vortex samples
4. Punch hole in lid of eppie and boil for 5 minutes at 95°C
5. Spin samples down with quick pulse and place back on ice
6. Assemble the gasket
   - Assemble the gels onto the gasket with the comb facing inside
   - Fill the gasket with running buffer – checking for leaks
   - Carefully remove the combs and rinse out wells with P200 pipette
7. Load ladder (4 µL BLUeye)
8. Load standard and samples
9. Place gasket into tank and fill with running buffer. Re-fill gasket if necessary
10. Attach lid (black on black, red on red) and plug into power pack
11. Run at 80 V for 10-20 minutes (until sample has entered gel)
12. Increase to 100 V and run until blue dye front reaches the bottom of the gel (~75 minutes)

Before its finished, prepare for transfer:

- Soak two pieces of Bio-Rad blotting paper and a Nitrocellulose membrane in transfer buffer for 2-3 minutes.

13. When finished, activate immediately
14. Activate stain-free properties of gel on ChemiDoc
    - Gel activation protocol, 2.5 minutes
    - If looking at a low molecular weight protein, activate with faint bands
    - When looking at faint bands, higher exposure time
15. Assemble the transfer sandwich on the cassette base (anode) by placing one piece of blotting paper on the bottom, then the membrane, then gel, and finally, the second blotting paper on top.
    - Use the blot roller to remove air from between the assembled layers.
    - Get excess liquid out by tilting the cassette and dabbing
16. Once the stacks are positioned in the cassette base, place the cassette lid on the base.
17. Slide the cassette (with the dial facing up) into the bay until it makes contact with the magnetic interlock and you hear a click. Cassettes can be inserted into the bays in any order, with or without power to the system.
18. SETTINGS FOR MACHINE:
    - Home menu → LIST button → Biorad pre-programmed protocols → MIXED MOLECULAR WEIGHT transfer protocol
19. To initiate the run, press the navigation button that corresponds to A:RUN for the cassette in the upper bay or B:RUN for the cassette in the lower bay.
20. After transfer, disassemble stack
    - Gel can be visualised to determine transfer efficiency if desired
    - Place paper towel in between the cassettes to soak up any excess liquid
21. Air-dry membrane
    - Hold in fumehood for quicker drying
22. Label membrane
23. Re-hydrate membrane in transfer buffer
24. Wash membrane in TBS-T for 5 minutes
25. Image transfer on membrane and acquire total protein image on Chemidoc
    - Stain-free blot setting
    - If using for normalisation, make sure image is clean and not over-exposed
26. Block membrane:
    - 5% milk (prepared in TBS-T) for 1-2 hours with gentle shaking
    - 10 mL Blocking buffer (Bio-rad) for 5 minutes
27. Wash membrane 3x for 5 min in TBS-T
28. Incubate membrane on primary antibody at 4°C overnight
    - Prepared in 50 mL centrifuge tube

- Dilute antibody in TBS-T to desired concentration
- Attach to rotators in walk-in fridge

Day 2

1. Retrieve membranes
    - Stored primary antibody in fridge/freezer
2. Wash membrane 3x 5 minutes in TBS-T
3. Incubate membrane on secondary antibody for 1 hour at RT
    - Prepared in 50 mL centrifuge tube
    - Dilute antibody in TBS-T to desired concentration, normally 1:10 000
4. Wash membrane 3x 5 minutes in TBS-T
5. Develop
    - Prepare minimum amount of ECL needed in eppie in 1:1 ratio
    - Place membrane on Chemidoc and check position
    - Add ECL to the desired area and roll to ensure even spread. Roll away excess ECL

    a. Expose using desired settings