UNIVERSITY OF HULL

Determination of the role of WSB-1 in breast cancer metastasis to the bone

By

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Abstract

Breast cancer is the second most common cause of female-related deaths in the United Kingdom with metastatic spread the main reason for treatment failure and cancer-related deaths. The bone is the most frequent site of secondary cancer, significantly decreasing patient's quality of life and survival. WSB-1 is a hypoxic-responsive E3 ubiquitin ligase which has been found to be upregulated in metastatic cells. Furthermore, studies investigating WSB-1 have found it to be involved in the progression of various tumour types, including metastatic breast cancer, but specific roles in bone metastasis have not yet been identified. This study aims to identify potential pathways in breast cancer bone metastasis.

In order to do this, first *in silico* bioinformatic analysis of available RNA-sequencing data from the lab for WSB-1 knockdown was performed to identify significantly regulated bone metastasis factors potentially downstream of WSB-1. These were then validated through qPCR, ELISA and western blotting after siRNA treatment. Further identification of clinical relevance was then explored through *in silico* analysis of publicly available breast cancer patient datasets for correlation in gene expression between *WSB1* and candidate genes.

Overall, in MDA-231-IV breast cancer bone homing cells, WSB-1 knockdown led to significant downregulation of *ICAM1, IL1B, MMP1, MMP9, TGFBR1, PTGS2* and *PTHLH*, which are all key genes associated with breast cancer and bone metastasis. Further analysis of IL1β protein levels was explored using western blotting and ELISA, however, no significant differences was found when the knockdown of WSB-1 was present. Finally, *in silico* analysis further validated the correlation between WSB-1 and these factors in breast cancer patient mRNA expression datasets.

To conclude, WSB-1 could be involved in the biology of breast cancer bone metastasis by enhancing the vicious cycle of bone metastasis, but further validation of the identified factors and their impact in bone metastasis biology needs to be investigated. Further work could validate WSB-1 to be used clinically as a prognostic biomarker, with high levels indicating to drive metastatic potential or a predictive biomarker with the potential to inhibit WSB-1 using IL1β antibodies, and gene inhibitors such as *ICAM1, MMP1, TGFBR1 and PTHLH*. With further research, these treatment options could be successful and therefore aid in increasing distant metastasis-free survival and potentially patient overall survival rates.

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Abbreviations

2D: two dimensional

3D: three dimensional

°C: degree Celsius

ANOVA: analysis of variance

B2M: β-2-microglobulin

BC: breast cancer

BRCA1: breast cancer 1, early onset

BRCA2: breast cancer 2, early onset

cDNA: complementary DNA

CO_2: carbon dioxide

COX2: cyclooxygenase 2

Ct: cycle threshold

CXCR4: C-X-C chemokine receptor type 4

DEPC: diethylpyrocarbonate

DKK-1: dickkopf-1

DMEM: Dulbecco's Modified Eagle Medium

DMFS: distant metastasis-free survival

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

ECM: extracellular matrix

ECS: elongin-Cullin-SOCS box

ELISA: enzyme-linked immunosorbent assay

EMT: epithelial to mesenchymal transition

ER: oestrogen receptor

ET1: endothelin-1

FBS: Foetal Bovine Serum

FC: fold change

FGF: fibroblast growth factor

HER2: human epidermal growth factor 2 receptor

HIF: hypoxia-inducible factor

HIPK2: homeodomain-interacting protein kinase 2

ICAM: intercellular adhesion molecule

IGF: insulin-like growth factor

IGF: insulin-like growth factors

IGFIR: insulin-like growth factor-I receptor

IL-21R: interleukin-21 receptor

IL: interleukin

JAK-STAT3: janus kinase/signal transducers and activators of transcription

JNK: c-Jun N-terminal Kinase

kPa: kilopascals

LMNB1: lamin B1

M-CSF: macrophage colony-stimulating factor

MAPK: mitogen-activated protein kinase

ml: millilitre

mm: millimetre

MMP: matrix Metallopeptidase

mRNA: messenger ribonucleic acid

mRNA: messenger ribonucleic acid

NK cells: natural killer cells

nM: nanometre

O_2: oxygen

O_2: oxygen

OPG: osteoprotegerin

OS: overall survival

PBS: Phosphate-Buffered Saline

PD-L1: programmed death-ligand 1

PDGF: platelet-derived growth factor

PGE2: prostaglandin E2

pH: potential of hydrogen

PHD: prolyl hydroxylases

PI3K: phosphatidylinositol 3-kinase

PKB: protein kinase B

PKB: protein kinase B

PR: progesterone receptor

PTHrP: parathyroid hormone -related protein

PTHrP: parathyroid hormone-related protein

PVDF: polyvinylidene difluoride

pVHL: von Hippel-Lindau tumour suppressor

qPCR: quantitative polymerase chain reaction

RANKL: receptor Activator of Nuclear Factor κ B Ligand

RHOA: ras Homolog Family Member A

RhoGD12: Rho GDP-dissociation inhibitor 2

RNA: ribonucleic acid

ROS: reactive oxygen species

ROX: carboxy-X-rhodamine

rpm: rotations per minute

RUNX2: runt-related transcription factor 2

SD: standard deviations

SDS-PAGE: sodium dodecyl sulfatepolyacrylamide gel electrophoresis

SEM: standard error means

Shh: sonic hedgehog

siNT: non-targeting siRNA

siRNA: small interfering RNA

siWSB-1: siRNA against WSB-1

SOCS: suppressor of cytokine signalling

SOCS: suppressor of cytokine signalling

SPARC: secreted protein acidic cystein-rich

TAK-1: transforming growth factor-β-activated kinase 1

TBS: Tris-buffered saline

TGFB1: transforming growth factor-β1

TNBC: triple-negative breast cancer

TNF: tumour necrosis factor

TNFB: transforming growth factor-beta

UTB: urea/Tris HCl buffer

V: volt

VCAM1: vascular cell adhesion molecule 1

VEGF: vascular endothelial growth factor

WSB1: WD Repeat and SOCS Box-Containing 1

µg: microgram

µl: microlitre

µM: micrometre

Chapter One: Introduction

1.1 Breast cancer overview

Breast, lung, and colorectal cancer are the most common cancer diagnoses in women in the United Kingdom (Figure 1), with breast cancer being the second most common cause of female cancer-related deaths (Cancer Research UK, 2022). Although being one of the United Kingdom's most deadly diseases, through screening, early diagnosis and improved treatments, the survival rates for 5 or more years after diagnosis are around 85% (Cancer Research UK, 2023).

1.1.1 Breast cancer subtypes

A main factor in determining breast cancer subtypes is hormone receptor expression, namely that of oestrogen receptor (ER) or progesterone receptor (PR), as well as that of human epidermal growth factor 2 receptor (HER2) expression (Table 1) (Waks and Winer, 2019). Gene expression-based assays and immunohistochemistry (IHC) based markers are often used to determine the subtypes of breast cancer (Gao and Swain, 2018). Luminal breast cancers are the most common subtypes of breast cancer to be diagnosed; however, research often focuses on HER2 and basal subtypes due to having a poorer prognosis (Wiechmann *et al*., 2009; Li et al., 2016).

Table 1: The three major subtypes of breast cancer according to receptor expression (from Zhao et al., 2020)

Breast cancer subtype	**ER**	**PR**	**HER2**
Luminal A	+	+	-
Luminal B	+	+	+
HER2+	-	-	+
Basal (TNBC)	-	-	-

ER: oestrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor, TNBC: triple-negative breast cancer.

The subtype **Luminal A cancers** are slow growing, due to the low levels of the nuclear protein and proliferation factor Ki-67 (Liang et al., 2020; Lombardi et al., 2021). Around 50-60% of breast cancer diagnoses are of subtype luminal A, where low proliferation levels mean that the prognosis is good and ER and PR positivity mean these tumours can be treated with endocrine therapy and chemotherapy (Loibl and Gianni, 2017).

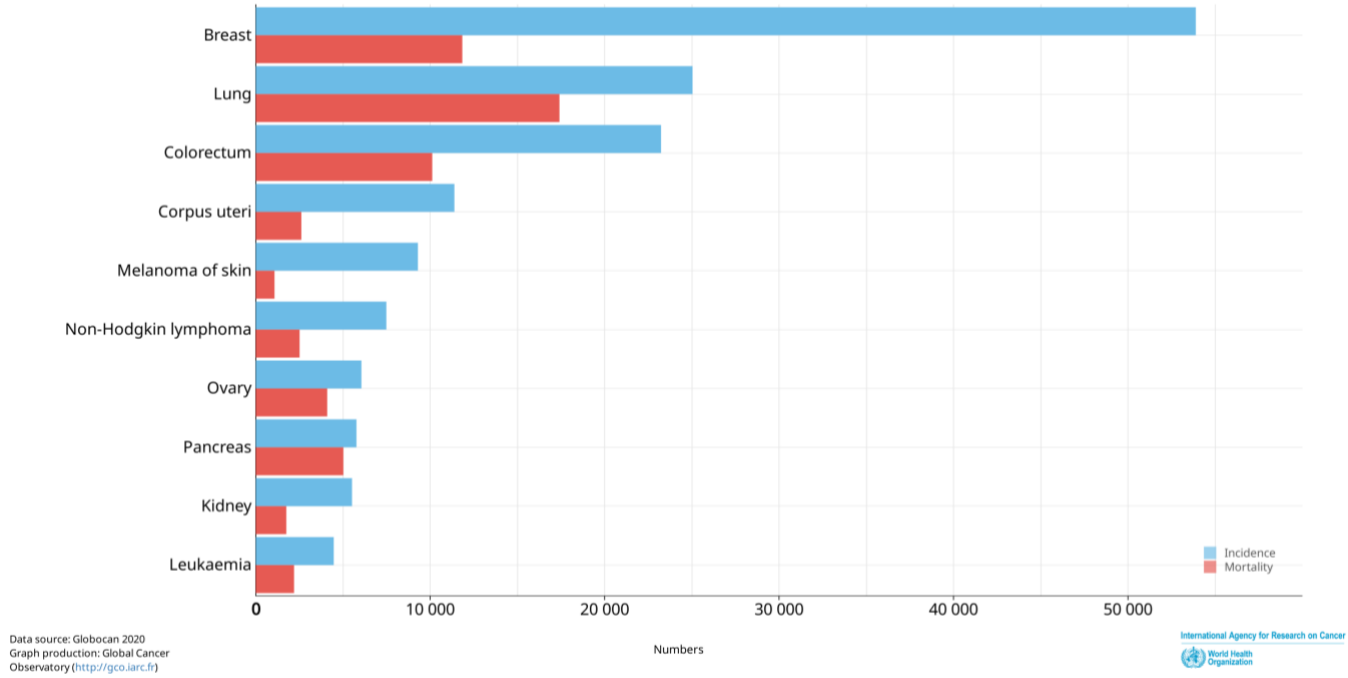


Figure 1: Estimated number of diagnosis cases and deaths in the United Kingdom, females of all ages (from Global Cancer Observatory, 2020).

In contrast, **Luminal B tumours** have high Ki-67 expression levels, resulting in faster proliferating tumours and therefore poorer survival rates (Erber and Hartmann, 2020). The Luminal B subtype can be further categorised by the expression of HER2, which can offer the option of anti-HER2 therapy treatment (see section 1.1.3). HER2 controls healthy cell division, although its amplification leads to uncontrolled division (Goutsouliak et al., 2020). **HER2+ cancers** were known to have poor survival rates due to high proliferation and metastasis to the brain (Patel et al., 2020).

TNBC is known as the most aggressive subtype and commonly metastasises to the bone, lung, and lymph nodes (Pellegrino et al., 2021). It corresponds to 15-20% of all breast cancers (Yao at al., 2019). Once TNBC becomes metastatic, fewer than 20% of patients survive four years post-diagnosis, compared to the other subtypes where survival is estimated at 40% four years after diagnosis (Heeke and Tan, 2021). The most common gene mutation associated with TNBC is the mutation of the tumour protein 53 (*TP53)* gene, accountable for 60-80% of TNBC diagnoses (Mitri et al., 2022). The mutation either occurs when the loss of *TP53* is present, resulting in the loss of regulators controlling proliferation and apoptosis, or a gain in the function responsible for proliferation, metastasis and drug resistance (Darb-Esfahani et al., 2016). 10-20% of patients diagnosed with TNBC have a genetic mutation involving the *BRCA1* and *BRCA2* genes, which can be inherited or arise from genetic or environmental factors (Forbes et al., 2019; Zhao et al., 2020). Wildtype *BRCA1* and *BRCA2* genes are tumour suppressor genes, responsible for checkpoints to control DNA damage repair, apoptosis, and transcriptional regulations (Song et al., 2020). Women carrying mutations in these genes are more than 75% likely to be diagnosed with breast cancer at an alarmingly young age, leading to many women receiving a bilateral prophylactic mastectomy (van Barele et al., 2022).

Subtypes often differ in their metabolic state, the expression of the enzyme responsible for fatty acid synthase has been shown to have connections with cancer development, recurrence, and poor prognosis. Research by Kim et al. (2015) presented that high expression of fatty acid synthase was more significant in HER2+ subtypes and lowest in that of TNBC. Although this study has 476 participants, only 10.5% were in the category of having the subtype TNBC, this may be a factor as to why in this study fatty acid synthase expression in TNBC was significantly less than the other subtypes. Although the study has this limitation, Yang et al., (2013) also observed that HER2+ breast cancer subtypes were strongly correlated

with fatty acid synthase, with findings showing that HER2 receptors interact with the expression of fatty acid synthase. However, this study would have aided in having a larger sample size of that of 100 cases.

1.1.2 Breast cancer staging

As well as which receptors are present, breast cancer is also categorised into stages from 1 to 4, each stage having varied survival rates (see Table 2). Women in stage 1 have a greater chance of survival due to early intervention (Saadatmand et al., (2015). Although more than 99% survive five years post-diagnosis, Wang et al. (2019) state that around 20-30% of those develop metastatic disease later in their treatment. Additionally, only 25% of women who fall into the stage 4 category will survive five years post-diagnosis (Cancer Research UK, 2020). Wang et al, (2019) studied the survival rates of people diagnosed with stage 4 breast cancer. For these, of 18,322 patients' bone-only metastasis accounted for 39.80% of patients. In this study patients with bone metastasis survived the longest, (34-37 months) compared to metastasis to the brain which was 5-10 months. Although patients with metastasis to the bone survived the longest, this type of disease can be detrimental to the patients' quality of life. With treatment plans likely for the rest of their lives they can experience high levels of pain, lack of energy, and a substantial amount of time in hospital and palliative care (Reed et al., 2012).

Table 2: Stages of breast cancer categorised to tumour size and affected areas (from Breast Cancer UK, 2023)

Stage	Tumour size	Affected areas	Survival rate after 5 years of diagnosis
Stage 1			
1A	< 2cm	None	<99%
1B	< 2cm	A few in nearby lymph nodes	
Stage 2			
2A	> 2cm	A few lymph nodes in the armpit or breastbone	<90%
	Between 2cm and 5cm	None	
2B	Between 2cm and 5cm	Nearby lymph nodes	
	Between 2cm and 5cm	A few lymph nodes in the armpit or breastbone	
	<5cm	None	
Stage 3			
3A	Any size	4-9 affected lymph nodes in the armpit or breastbone	<70%
	>5cm	1-3 affected lymph nodes in the armpit or breastbone	
3B	Any size	Skin Chest wall Up to 9 lymph nodes in the armpit or breastbone	
3C	Any size	Skin Chest wall Up to 10 lymph nodes in the armpit or breastbone Lymph nodes close to the collar bone	
Stage 4			
	Any size	Metastasised to areas such as brain, lung, liver and/or bone	<25%

1.1.3 Breast cancer treatment

Treatment for breast cancer varies widely on the patient and the stage and subtype of cancer. The main treatment plans include and may have a combination of surgery, chemotherapy, radiotherapy, hormone-related therapies, and targeted therapies (See Table 3).

Table 3: Effective treatment plans dependent on the breast cancer subtype (Balma et al., 2022)

Breast cancer subtype	**Endocrine therapy**	**Anti-HER2 therapy**	**Chemotherapy/radiotherapy**
Luminal A	+		+
Luminal B (HER2-)	+		+
Luminal B (HER2+)	+	+	+
HER2+		+	+
Basal (TNBC)			+

Surgery treatment can mean local or a loss of the whole breast. Breast conservation surgery can be offered to the patient if the tumour size is small and is localised, this type of surgery offers quicker recovery time and less intervention. Golshan et al. (2015) found that breast-conserving therapy had a 91% success rate of 53 patients offered the treatment. Interestingly, Badwe *et al*., (2015) found no or little difference in the life expectancy of patients who underwent surgery or not, this study is however flawed due to the recruitment of patients who only had a life expectancy of one year at the start of the study.

Post-surgery treatment can include **radiotherapy** targeted at the local area in the breast and lymph nodes. Radiotherapy uses high-energy radiation targeted at the site of the tumour, causing damage to the DNA structure through breaks in the DNA strands (Wengner et al., 2020). Radiotherapy although still distressing can have a lesser effect on the body than chemotherapy, improving quality of life and reducing side effects (Recio-Saucedo et al., 2016).

Chemotherapy can be given before surgery as well as after, pre-surgery chemotherapy is called neoadjuvant and post-surgery, adjuvant chemotherapy. Chemotherapy is commonly administered intravenously (IV) but can be given orally or directly, as a cream. Chemotherapy

damages the DNA of cells that are going through mitosis. Neoadjuvant therapy is treatment targeted at reducing the size of the tumour before surgical removal, this can be in the form of chemo or radiotherapy (Masood, 2016; Wang and Mao, 2020). Guestini et al., (2019) found that patients with TNBC that were given neoadjuvant therapy were shown to have an increase in the protein expression of ABCC1, a well-known chemo-resistant factor (Emmanouilidi et al., 2020).

Wildtype cells have hormone receptors to allow for normal pathway function and survival. Certain types of breast cancers are hormone-responsive, meaning that for survival and proliferation, they must have hormone receptors that hormones such as progesterone or oestrogen bind to. **Hormone receptor targeted therapy** is a medication that inhibits hormone receptors, prevents hormones from binding to the receptors, and inhibits responsible pathways for proliferation and survival (Ali *et al*., 2016). Hormone receptor targeted therapy is only effective for cancers that have hormone receptors such as luminal and HER2 subtypes. An example of this type of drug is tamoxifen. Tamoxifen works by engaging with the oestrogen receptor in the tumour cells, blocking the receptors from oestrogen and inhibiting that pathway, thereby preventing cell division (Manna and Holz, 2016). This type of medication has been a popular choice for patients for around 40 years and has reduced mortality rates by up to 30% (Ali *et al*., 2016). Despite this success, tamoxifen resistance is a limitation, with around 40% of patients having this treatment acquiring resistance (Hultsch et al., 2018).

Targeted therapeutics are drugs that target specific proteins and genes that are changed within tumour cells. Small-molecule drugs work by penetrating the cells, binding to proteins, and inhibiting their expression (Cao and Zhang, 2019). Trastuzumab is a licensed targeted therapy drug, specifically a monoclonal antibody that is used in the early stages of breast cancer, it works by targeting HER2 proteins on the cell surface and therefore blocking proteins from attaching and allowing the cell to go through mitosis. Due to trastuzumab targeting HER2 receptors, this drug only specifically works on cancer types that are HER2 positive. The drug trastuzumab was the most used in the 90s (Incorvati et al., 2013), recent research has shown that using this drug in conjunction with others has significant effects on survival rates. Pertuzumab, binds to different regions of the HER2 protein receptor that trastuzamab does, making it a more effective treatment. An example of this can be seen in studies by Lui et al. in 2022 and Piccart et al. (2021), where adding Pertuzumab to the treatment plans alongside

trastuzumab, significantly improved invasive disease-free survival (P=0.02). Therefore, concluding that recent research and clinical trials have had a positive impact on patient survival rates.

Tian et al. (2022) studied a modified version of trastuzumab that specifically targets bone metastasis, they modified the drug with bone-homing peptide sequences that were able to access and be effective in the niche environment of the bone. They found that in the group of mice receiving the modified version of trastuzumab, the tumour lesion on the bone within the mice was significantly smaller than in the trastuzumab only group ($p < 0.0001$) and that the mice were 51% more likely to have survived at the end point of the 52 day experimental trial. Although the apparent success of the experiment, limitations were seen due to the small sample size (8 mice per group). Although treatment options such as chemotherapy and targeted therapy offer good survival rates five years post-diagnosis, they can impact fertility and prompt early menopause, having a detrimental effect on a patient's quality of life and wellness (beak et al., 2023).

Advances in recent medicine have allowed for more personalised treatment plans such as immunotherapy. These include but are not limited to immune checkpoint inhibitors and T-cell transfer therapy. Immune checkpoint therapy works by blocking certain immune checkpoint proteins on the cancer cell such as PD-L1 (programmed death-ligand 1) (Gaynor et al., 2022). Blocking these proteins allows the cancer to not go undetected by the immune system and consequently cytotoxic lymphocytes detect and attack the cancer cells, causing apoptosis (Thomas et al., 2021). A study by Cortes et al. (2022) investigated TNBC-diagnosed patients, the method contained a range of cancerous stages and took into consideration the programmed death ligand 1 (PD-L1) expression. Cortes found that patients with a higher expression of PD-L1 were overall surviving longer than the group with less PD-L1 levels. It was also found that the pembrolizumab control group were significantly living longer than the placebo group in patients with higher PD-L1 scores. However, patients that had lower expressions were not significant in their differences in survival rates, with the mean results being 17.2 for the pembrolizumab group and 15.5 months who had the placebo.

Thomas et al. (2021) and Cortes et al. (2022) describe immune checkpoint inhibitors as a less harmful type of therapy compared to chemotherapy and for metastatic breast cancer, immunotherapy is seen as the future of treatments. Although these studies' results look

satisfactory, they do highlight that a deeper knowledge of gene expression and the personalisation of combined therapies could contribute to the future of cancer treatments.

1.2 Metastatic Breast Cancer

1.2.1 Overview of Breast Cancer Metastasis Sites

A hallmark of cancer is the ability to invade tissue and metastasise. This has been proposed as the main reason for treatment failure and is responsible for more than 90% of cancer-related deaths (Hanahan and Weinberg, 2011; Chen et al., 2018; Wang et al., 2021). The chances of breast cancer turning metastatic is between 20 and 30%, the bone being the most frequent site of metastasis (30-60%), followed by the lung (21-32%), liver (15-32%) and brain (4-10%) (Tahara et al., 2019; Wang et al., 2021). Newly formed blood vessels are often made of discontinuous endothelium, causing them to leak nutrients and oxygen, this weakness causes metastatic cells to pass through vessel walls with ease (Jing et al., 2019). Furthermore, healthy epithelial cells have adhesion regulators such as E-cadherin, regulations like this help to bind the same kind of cells to one another, creating the extracellular matrix. In cancerous cells proteins like E-cadherin are lost or corrupted, playing a key role in the involvement of metastatic dissemination (Borsig et al., 2019). Without the ability to adhere, cancer cells often enter the bloodstream and invade local tissue or lymph nodes or metastases to distant parts of the body (see Figure 2). Interestingly, although there is an initial loss of E-cadherin when cells metastasise, adherence proteins are re-expressed, enabling tumour cells to interact in their secondary site and microenvironment (Wells et al., 2008).

It is worth noting that specific subtypes favour certain metastatic sites more than others, this is known as organotropic metastasis (Chen et al., 2018). Cancer cells can reprogram their metabolic state to exhibit different metabolic phenotypes depending on their receptor state (Wang et al., 2021). Arciero et al. (2019) stated that ER+ and HER+ subtypes were more likely to metastasise to the bone but less likely to the brain, liver, and lung, compared to subtypes which were ER- and HER2+. Of a study containing 243,896 patients looking into metastasis sites involving all subtypes, in 8848 cases, bone metastasis was the most found site and brain metastasis was next within 1000 patients (Wu et al., 2017). It is believed that BC cells that have elevated expressions of cyclooxygenase 2 (COX-2) tend to home to the bone, high levels

of COX-2 are also associated with larger tumour sizes, high p53 expressions and overall poor survival (Ristimäki et al., 2002). COX-2 expression is not normally observed in healthy adult cells, it is however expressed in cells that are under high inflammatory stress, such as cancerous cells. Genes on the chromosome that are responsible for the transcription of COX-2 are stimulated by cytokines such as the interleukin IL1B and transforming growth factors-beta (TGF-b) (Venetis, et al., 2021). These factors have important roles in the microenvironment of the cancer, which is why several studies suggest that COX-2 inhibitors could reduce the risk of bone metastasis in the later stages of breast cancer (Regulski et al., 2016; Wu et al., 2017).

Genetics can also influence metastatic sites with a study by Song et al., (2020) finding that most *BRCA1* mutation carriers experienced secondary sites to the lung and distant lymph nodes and the bone being the secondary site for B*RCA2* mutation carriers.

Breast cancer cells are said to contribute to the pre-metastatic niche, the ability to secrete factors through extracellular vesicles that reach the secondary organ, priming the extracellular matrix and allowing for hostile environments to become favourable conditions in which the cancer cells will thrive (Chen et al., 2018; Yuan et al., 2021). This theory, from 1889 is often called the “seed and soil” theory, creating fertile soil for the seed. Studies show that the expression of vascular cell adhesion molecule 1 (*VCAM1*), intercellular adhesion molecule 1 (*ICAM1*), and interleukin 6 (*IL6*) by breast cancer cells facilitates bone degradation in preparation for metastatic cancer (Massagué and Obenauf, 2016). As Cancer cells can often reoccur months or years after the primary tumour is removed (Coleman et al., 2020), future research is needed to gain an insight into whether primed secondary environments stay favourable until metastasis has occurred or if they return hostile. Furthermore, understanding the pre-metastatic niche could lead to therapeutic strategies to identify and prevent the event of metastasis.

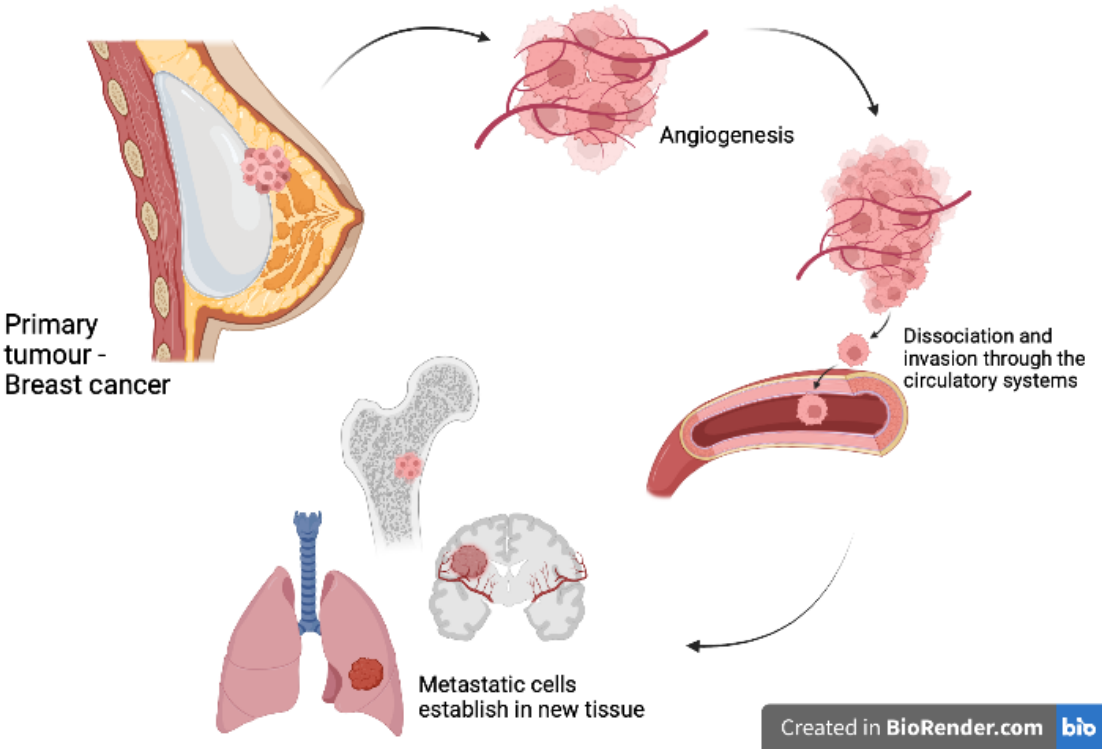


Figure 2: A flow diagram of the stages of cancer metastasis from the primary site to the secondary site (Figure created using BioRender)

1.2.2 Bone metastasis: microenvironment and signalling

Metastasis to the bone significantly decreases the patient's quality of life and survival, which is why it is extremely important to research this topic thoroughly. Bone metastasis is a severe condition due to the pain it can cause through fractures, spinal compression, cranial nerve palsies, and suppression of bone marrow function (Coleman *et al.*, 2020). Price et al. (2016) hypothesised that C-X-C chemokine receptor type 4 (CXCR4) interacts with breast cancer cells as one of the main niches of how tumour cells home to the bone. The research discovered that E-selectin, a hematopoietic vascular gateway, is critical for entry into the bone microenvironment and that interactions with CXCR4 anchor the tumour cells to this secondary site. Most importantly these findings offer an insight into how breast cancer cells home in the bone and a potential treatment option to avoid the emergence of relapsed disease. Several factors have been reported to have major significance in the bone metastasis cascade. A summary of these can be seen in table 4.

The skeletal system continuously remodels throughout life, a balance of bone-forming osteoblasts and bone-resorbing osteoclasts. Once the osteoblasts have come to the end of the bone formation cycle, they become trapped, maturing into osteocytes (Tahara et al., 2019). Osteocytes promote homeostatic maintenance and secrete biochemical signals when under stress, osteocytes are also said to be the main cytokine receptor for activation of Nuckear Factor-κB ligand (RANKL) (Marahleh et al., 2019; Kim et al., 2020). On the surface of osteoclast precursors lies the receptor to RANKL called RANK, this interaction causes the osteoclast to differentiate into bone-resorbing osteoclasts (Tahara et al., 2019). Changes in the bone microenvironment due to the presence of metastatic tumour cells unbalance the dynamic and create a vicious cycle that further promotes tumour progression (Figure 3) (Tahara et al., 2019).

Breast cancer cells metastasise to the secondary site of the bone through the bloodstream, there based on the type of mechanism lesions can be either osteoblastic or osteolytic (Goodman et al., 2015). In osteoblastic lesions, ET1 secretions by the tumour cells inhibit the expression of DKK-1, decreasing osteoblastic differentiation, and favouring bone formation. The opposite can be seen in osteolytic lesions. Cytokines and chemokines such as PTHrP, IL1β, IL6, IL11, PGE2, TNF and M-CSF are secreted by the tumour cells.

Table 4: Genes associated with breast cancer and bone metastasis

Gene	Role relating to breast cancer	Funtion in bone metastasis
TGFB1 (Tranforming Growth Factor Beta 1)	Cell motility, invasion, and metastasis (Hachim et al., 2018)	Produced by osteoblasts and received by the cancer cells. Also binds to SMAD2/3 and SMAD4 to promote invasion and angiogensis (Trivedi et al., 2021)
TGFBR1 (Tranforming Growth Factor Beta Receptor 1)	Accountable for providing instruction to make growth factor-beta receptor type 1 (Moore-Smith and Pasche, 2011)	Binds to and activates downstream receptor-regulated pathways such as SMADs, RAS, JNK and RHOA (Agyemang et al., 2022)
TNFRSF11B (RANK) (TNF Receptor Superfamily Member 11b)	Involvement in multiple hallmarks of cancer, including tumour survival, EMT, angiogenesis, invasion, and metastasis (Wang et al., 2022)	Expressed by the tumour cell, binding to RANKL, inhibiting the maturation and activation of pre-osteoclasts (Weichhaus et al., 2015)
RAP1A	Cell adhesion, migration, and survival (Zang et al,. 2023)	Expressed by the tumour cell and is a key regulator for lysophosphatic acid, leading to cell migration and invasion (Alemayehu et al., 2013)
IGF1 (Insulin Line Growth Factor 1)	Increases cell survival and proliferation	Produced by osteoblasts, chondrocytes, osteoclasts, and osteocytes. Believed to be the cross talk between the bone and metasisised cancer cells, activating IGFIR, Akt and RANKL pathways (Hiraga et al., 2012)
ADAMTS1 (ADAM Metallopeptidase With Thrombospondin Type 1 Motif 1)	Promotes osteoprotegerin by shedding tumour-derived epidermal growth factors (Lu et al., 2009)	Partakes in the releasse of EGF-like ligands, stimulating the production of osteoclasts in turn activating RANKL and OPG (Casimiro et al., 2012)
MMP1 (Matrix Metallopeptidase 1)	Promotes osteoprotegerin by shedding tumour-derived epidermal growth factors (Lu et al., 2009)	MMP1 prodoce collagen fragments which are responsible for the mutation of osteoclasts (Eck et al., 2009)
MMP9 (Matrix Metallopeptidase 9)	Produced by osteoclasts and immunity cells, facilitating in tumour invasion and proliferation (Liu et al., 2016)	Activate TGFb pathways also able to destroy collagen type 1, 4 and 5 leading to bone degrdation (Liu et al., 2016)
IL1BETA (Interleukin 1 Beta)	Codes for the interleukin IL1β. Upregulation induces OPG secretion awakening dormant tumour cells (Tulotta and Ottewell, 2018)	Secreted by tumour cells, osteoblasts and bone marrow cells, mediating angiogensis by inducing VEGF (Zhou et al., 2022)

IL6 (Interleukin 6)	Stimulates the production of osteoclasts (Hao et al., 2017	Secreted by the tumour cell, increading production of PTHrP, inducing expression of RANKL, activating osteoclasts and promote angiogensis (Ara and DeClerck, 2010)
IL8 (Interleukin 8)	Has a direct impact on osteoclastogenesis and bone resorption	Secreted by the tumour cell, effecting osteoblasts, increasing osteoclastogensis and potentially through an up-regulation of RANKL (Bendre et al., 2002)
IL11 (Interleukin 11)	Suppressed the activity of osteoblasts (McCoy et al., 2013)	Can be secreted by the tumour cells or stimulate osteoblasts to secrete the interleukin (McCoy et al., 2013)
CXCL8 (C-X-C Motif Chemokine Ligand 8)	Contributes to mammary tumour-induced osteolysis, aids in tumour proliferation, migration and bone bestruction (Sharma et al., 2019)	Triggers the JAK-STAT3 pathway, promoting tumourigenesis and osteolysis (Johnstone et al., 2015)
CSF1 (Colony Stimulating Factor 1)	Induces osteolysis	A Cytokine released by osteoblasts and tumour cells to induce pre-osteoclasts to develop into osteoclasts (Kang et al., 2019)
ICAM1 (Intercellular Adhesion Molecule 1)	Plays a part in intercellular adhesion and migration (Chen et al., 2022)	Expressed on the tumour cell surface (Chen et al., 2022)
PTHLH (Parathyroid Hormone Like Hormone)	Codes for the secreted factor PTHrP. Known to supress tumour development and invasion in early stages of cancer. Has also been identified to play a key role in the emergance of dormant cells in the bone (Martin and Johnson, 2021)	Expressed by tumour cells and binds to osteoblast cells enhancing RANKL production and osteoclast formation (Wang et al., 2014)
PTGS2 (Prostaglandin-Endoperoxide Synthase 2)	Often upregulated in breast cancer cells, believed to play a part in the migration and invasion and increase cell apoptotic resistance (Zhang et al., 2018)	Upregulated by the TAK-1 pathway and increases osteoclast formations by increasing *PGE2* production by the tumour cells (Li, et al., 2008)
VEGFA (Vascular Endothelial Growth Factor A)	Stress promotes cell intravastion into myphatic vessels through *VEGF* and *COX2* mechanism	Target gene for osteoblasts, stimulating OPG production promoting tumour angiogensis and invasion (Augustine et al., 2014)

This stimulates osteoclast differentiation. RANKL can be seen to be expressed by the tumour cells and osteoblasts, RANKL binds to RANK receptors, further stimulating the cascade to favour osteoclast formation. Ultimately leading to enhanced bone resorption and the release of metalloproteases, HCl and matrix-embedded growth factors (e.g., IGF-1, TGF-β, FGF and PDGF), promoting cancer cell proliferation and tumour progression (Venetis, et al., 2021).

There are several main signalling pathways involved in the cycle of bone metastasis. Here the RANKL-RANK pathway will be described in steps with mentions of additional signalling pathways. Firstly, seeded tumour cells secrete parathyroid hormone -related protein (PTHrP) and IL6 which stimulate the osteoblasts and osteocytes to produce RANKL (Shah et al, 2023). PTHrP is found to be upregulated and secreted by primary tumour cells in 60% of breast cancer cases. PTHrP expression here contributes to cell proliferation and apoptosis resistance (Soki et al., 2012). Importantly, PTHrP has also been shown to be a significant factor in promoting the emergence of dormant breast cancer cells in the bone with secretion levels 90% elevated in bone metastasic cells. Within the bone microenvironment, the secretion of PTHrP by the tumour cells activates osteoblasts, releasing growth factors and stimulating other pathways and therefore promoting a vicious cycle between the environment and the breast cancer cells in the bone matrix (Martin and Johnson, 2021).

After PTHrP has stimulated the production of RANKL through osteoblasts and osteocytes, RANKL then stimulates osteoclasts, causing bone resorption (Marahleh et al., 2019). Furthermore, the act of bone resorption stimulates the secretion of growth factors, such as TGF-β, fibroblast growth factors, runt-related transcription factor 2 (RUNX2) and IL-6 into the microenvironment and through canaliculi where then the growth factors are utilised by the tumour cells, promoting cancer cell proliferation and this further stimulating osteoclast activity, creating the vicious cycle (Mishra et al., 2011). The secretion of TGF-β is known to play an important role in the early stages of bone metastasis, as well as accelerating the cycle of tumour growth and epithelial-mesenchymal transition, it has also been shown to suppress the immune response by enabling cancer cells to escape immune checkpoints and promoting further tumour progression (Trivedi et al., 2021).

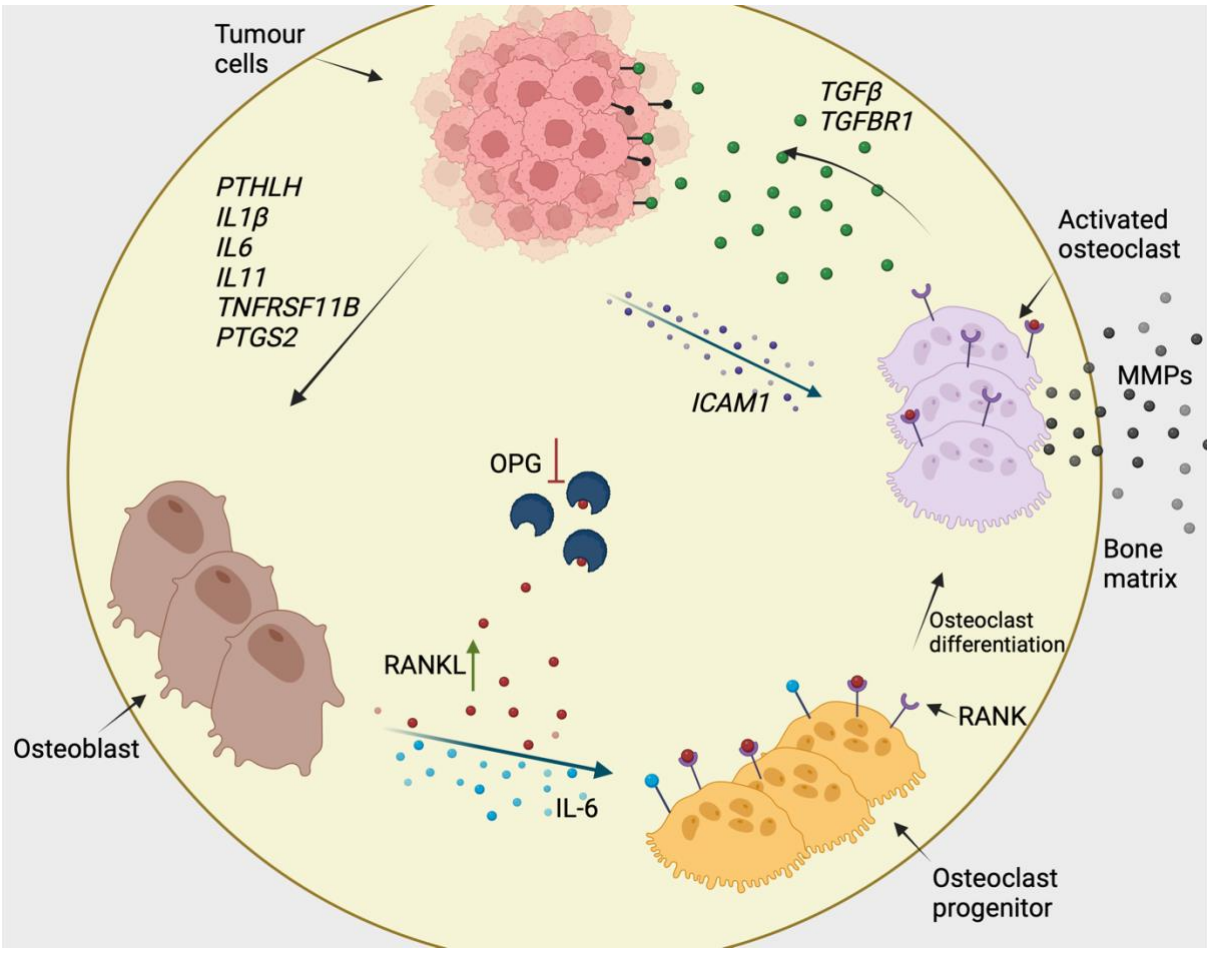


Figure 3: Breast cancer bone metastasis formation and vicious cycle (Figure created using BioRender).

Abbreviations: *PTHLH*, parathyroid hormone like hormone; IL, interleukin; *TNFRSF11B*, TNF receptor superfamily member 11b; *PTGS2*, Prostaglandin-Endoperoxide Synthase 2; *TGF*β, transforming growth factor-β; *TGFBR1*, Transforming growth factor-beta receptor type 1; MMP, matrix metalloprotease; *ICAM1*, intercellular adhesion molecule 1; OPG, osteoprotegin; RANKL, receptor activator of nuclear factor kappa-B ligand; RANK, receptor activator of nuclear factor kappa-B

It has also been well-researched that TGF-β can play a key role in chemotherapy resistance, targeted therapy and immunotherapy by activating alternative survival pathways and promoting anti-apoptotic signalling (Zhang et al., 2021). RUNX2 has been shown to upregulate TGF-B but to also have a direct influence on matrix metalloproteinases 2, 9 and 13, leading to the activation of bone destruction and cell migration through the degradation of the extracellular matrix (Koujan et al., 2015; Vishal et al., 2017).

Normal breast tissue has a stiffness of 3-16 kPa (McGarry et al., 2012), research by Shah et al, (2023) has shown that bone metastatic breast cancer cells express a higher significance rate of PTHrP and IL-6 when they are located in stiff areas such as the trabecular bone which is 4-80MPa (Shah et al., 2023). Breast cancer cells in bone metastasis are most commonly found within trabecular bones, further enhancing the adaptations of the tumour cells (Allocca et al., 2019). Another growth factor signalling pathway of bone metastasis is insulin-like growth factors (IGF) (Song et al., 2022). The IGF pathway is expressed in healthy bone tissue and plays an important role in regulating growth development by promoting proliferation and osteoclastogenesis. Breast cancer cells favour this pathway by expressing high levels of IGF type 1 receptors to which the IGF bind to (Christopoulos et al., 2015).

Furthermore, IGF is known to activate RANKL and protein kinase B (PKB), once activated PKB is involved in the hallmarks of cancer, such as; cell survival, proliferation and metabolism. Studies involving PKB knockout in mice have shown that this mechanism has a significant effect on osteoblast differentiation, results indicating that this signalling pathway interacts with transforming growth factor-β1 (*TGFB1*)-induced osteoblastic differentiation, acting as a switch (Suzuki et al., 2014). Elevated levels of *TGFB1* are known to promote angiogenesis and stimulate the production of extracellular matrix, creating perfect conditions for cell proliferation and migration (Bahhnassy et al., 2015). The overexpression of *TGFB1* has been shown to hinder osteoblastic differentiation (Xu et al., 2020), remarkably the ability of PKB to act as a switch, speeds up the cycle.

Other factors that affect the cycle are interleukins and Matrix metalloproteinases (MMP) (Koujan et al., 2015). Interleukins are small secreted signalling proteins, found to be expressed in healthy cells but additionally expressed by tumour cells to aid in their survival (Dinarello, 2015). Of all the interleukins, IL-6, IL-8, IL-1β, and IL-11 are shown to have significant roles in

bone metastasis specifically tumour proliferation and bone destruction, with higher levels of expression correlating with poorer prognosis and survival time (Knüpfer and Preiß, 2007; Yu et al., 2009; Salamanna et al., 2019). IL-6 has been shown to have involvement in many pathways such as Janus kinase, signal transducer and Jagged1. Interactions between Il-6 and Jagged1 were studied by Sethi et al., (2011), it was found that osteoblasts are receptors of Jagged1 and with this interaction, IL-6 proteins were released, promoting tumour cell proliferation. Furthermore, the expression of ILs increases RANKL signalling by stimulating osteoblasts to produce RANKL receptors, therefore enhancing the pattern for bone metastasis (Haider et al., 2021). Similarly to the interleukins, high expression levels of MMPs have also been significantly associated with poorer survival in patients with breast cancer bone metastasis (Ni et al., 2014). A study by Casimro et al. in 2013, saw that knocking down the expression of *MMP1* induced by RANKL found tumours in the bone significantly smaller than the control and a decrease in osteoclastogenesis, *MMP1* was found to be an inducer of osteoclastogenesis and therefore lead to a worse patient outcome.

As well as favourable extracellular matrix mechanisms which enhance tumour migration, research into hypoxic conditions has also indicated a strong influencer in the metastatic spread of patients whose primary tumours are poorly oxygenated and therfore increased risk of mortality (Gikes et al., 2014). Hypoxia-inducible factor (HIF) is the main transcription factor responsible for reprogramming cancer cells by regulating the expression of multiple genes involved in tumour progression (Petrova et al., 2018).

1.3 Tumour Hypoxia

1.3.1 Overview of tumour hypoxia

Oxygen levels vary within organs, however, tumour oxygen levels are often below 2% and even become near anoxic (<0.02% O_2) (Hompland et al., 2021). Hypoxic conditions within the tumour are caused by rapid tumour growth and cellular proliferation, coupled with increased metabolic demands and the occurrence of non-productive vasculature systems (see Figure 4) (Singh et al., 2017). The occurrence of regions of hypoxia is linked to poor patient prognosis, due to a combination of increased angiogenesis, metabolic switch, immune evasion, therapy resistance, and increased metastatic potential (Rankin et al. 2016). Specifically, hypoxia

biology has been shown to drive bone metastasis, increase tumour aggressiveness and have a detrimental impact on patient outcomes (Lundgren et al., 2007). Studies have shown that in bone metastasis reactive oxygen species (ROS) production is more than doubled in cancer cells in hypoxic conditions from 2-21% O_2 and have a four-fold increase at 0.5% O_2 (Li et al., 2016; Aggarwal et al., 2019). Interestingly in bone metastasis, the increased levels of ROS have been shown to promote the formation and activation of osteoclasts (Nishimura et al., 2016). ROS can cause cell survival or apoptosis through oxidative stress, resulting in enhanced cytotoxicity and apoptosis, it has also been shown to cause multidrug resistance (Jing et al., 2019).

Hypoxia is known to drive the angiogenic switch creating non-productive vasculature, which is often irregular and leaky (Chappell et al., 2019). Tumour cells in hypoxic stabilise HIF proteins and trigger the synthesis and release of proangiogenic factors such as VEGF (Aguilar-Cazares et al., 2019). The secretion of VEGF is needed to reach the receptors on nearby capillaries to stimulate angiogenesis. VEGF released from the tumour cells however struggle to penetrate through the ECM (Afonina et al., 2017). Nearby macrophages and mast cells are signalled by the tumour cell to release MMPs, MMPs then degrade the ECM, and thus releasing VEGF to bind to receptors on the endothelium cells located on the capillaries (Abdulkhaleq et al., 2018). The binding of the receptor stimulates the endothelium cells to become more permeable, releasing more MMPs and urokinase plasminogen activator (UPA), leading to further degradation of the ECM. The lack of ECM stimulates the release of TGF-B, platelet-derived growth factor (PDGF), angiogenin and oncoprotein (Filippi, 2016). The increased uptake of VEGF activates the mitogen-activated protein kinase signal transduction pathway instructing endothelial cells to proliferate, the breakdown of ECM allows for this movement (Dhillon et al., 2007). The release of proangiogenic factors also signals the recruitment of endothelial progenitor cells from the bone marrow, accelerating the process (Rana et al., 2018).

Hypoxia-induced tumour cells can reprogram their energy metabolism through aerobic glycolysis to limit the amount of oxygen needed (Jeong et al., 2019). This type of metabolic switch is known as the Warburg effect, a commonly known hallmark of cancer but not exclusively regulated by hypoxia (de la Cruz-López et al., 2019; Kung-Chun Chiu et al., 2019). Healthy cells need oxygen to undergo oxidative phosphorylation (OXPHOS) to produce

adenosine triphosphate (ATP) energising the cell (Tang et al., 2021), however, due to the lack of oxygen in hypoxic conditions, these cells are forced to change their energy metabolism to cater for this, known as glycolysis (Jeong et al., 2019). Healthy cells consume glucose and produce pyruvate and a small amount of lactose, cancer cells behave the same but produce a major amount of lactate due to glycolysis (Certo et al., 2021). This is used as a fuel source for nearby cancer cells. The excess lactate is extruded by transporters such as monocarboxylate transporter 4 (MCT4) and carbonic anhydrase IX (CAIX), which leads to an acidic microenvironment which can further stimulate angiogenesis and tumour growth and can lead to therapy resistance (Végran et al, 2011). Recent findings by Geeraerts et al. (2021) discovered that macrophages in the acidic tumour environment develop the ability to inactivate the immune cells that would have recognised and destroyed the cancerous cells.

One key feature of tumour hypoxia is the resistance to chemotherapy and radiotherapy (Ng et al., 2018). A seminal study from 1950 by Gray et al. showed hypoxic cells at an oxygen and nitrogen atmosphere pressure at 1, needed a three-fold higher dosage of radiation to be killed when compared to those in oxic conditions (20-21% O_2) (Graham and Unger, 2018). The oxygen levels at which this effect occur, usually <0.2% oxygen, is noted as radiobiological hypoxia (Busk et al., 2020).

This effect is mediated by the need for the presence of oxygen to mediate the accumulation of lethal damage to DNA caused by ionising radiation due to the lack of long-lived reactive oxygen radicals (Walsh and Kolb, 2014). In terms of chemotherapy resistance, hypoxia drives this through multiple mechanisms. One is that some chemotherapy agents require oxygen to cause DNA damage, employing oxygen molecules, such as alkylating agents through the nucleotide excision repair pathway (Chiorcea-Paquim et al., 2023). Furthermore, cells within the hypoxic areas proliferate slowly, effectively making them non-target areas for conventional chemotherapy drugs which target rapidly dividing cells (Jing et a., 2019). Finally, hypoxia can drive the expression of multi-drug resistance factors that can increase drug efflux (Jing et al., 2019).

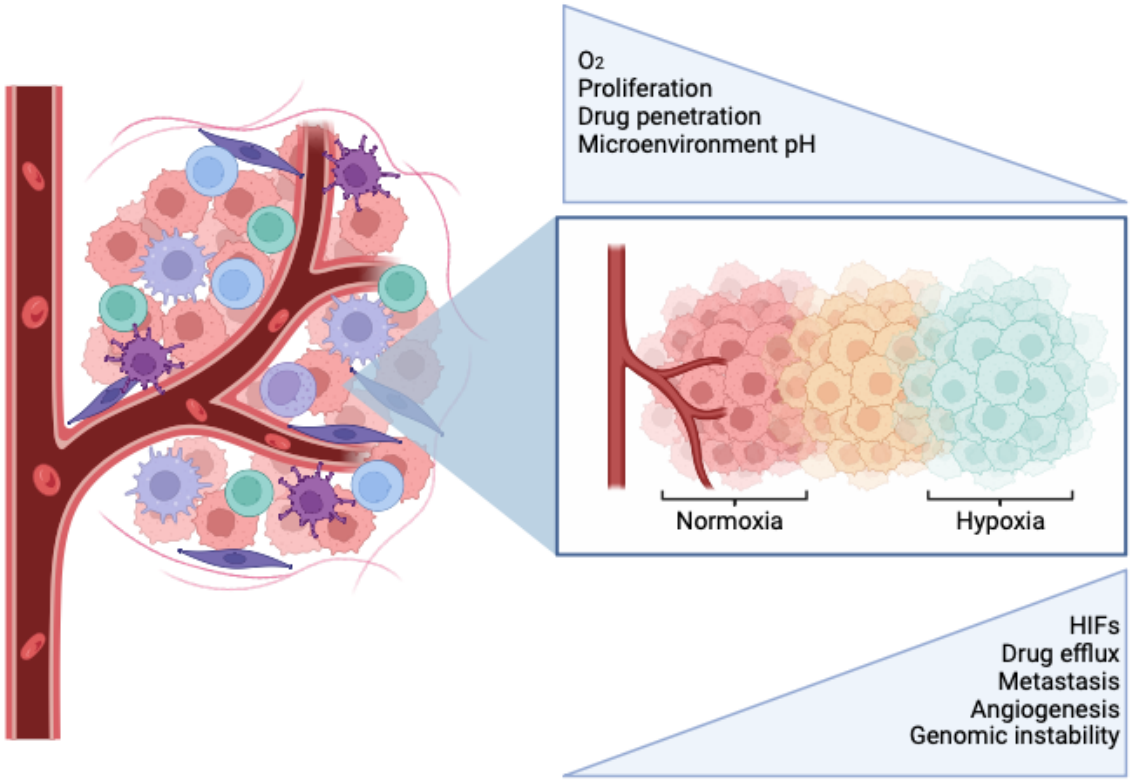


Figure 4: Hypoxic tumour regions present further away from blood vessels. (Figure created using BioRender)

Hypoxic signalling promotes the formation of a premetastatic niche and has been recognised as a key molecular feature that governs the metastatic potential (Rankin et al., 2016). Hypoxia stabilises hypoxia-inducible factors (HIFs) 1 and 2, this allows cells to adapt to the hypoxic stress, activating gene expression which is responsible for angiogenesis, glycolytic metabolism, migration, and invasion (Gaete et al., 2021). The stabilisation of HIFs allows for signalling to directly upregulate the expression of chemokines and cytokines responsible for the recruitment of macrophages and mesenchymal stem cells, in turn supporting the tumour cell in metastasis (Chaturvedi et al., 2014). Furthermore, the enhancement of HIF signalling results in an upregulation of MMPs, cathepsins, and lysyl oxidases which further support the remodelling of the tumour matrix, aiding in metastasis (Sudhan et al., 2013).

1.3.2 Hypoxia Inducible Factors (HIFs)

The cellular response to hypoxic conditions is primarily regulated by the Hypoxia Inducible Factors (HIFs) transcription factor family (Kumar and Choi, 2015). HIFs are a family of heterodimeric transcription factors that are critically upregulated in hypoxic conditions, having a knock-on effect on the expression of multiple genes that are involved in the progression of the tumour and has been highly linked to poor prognosis outcomes (Walsh and Kolb, 2014; Wigerup et al., 2016; Jing et a., 2019). HIFs consist of two subunits: HIF-α (HIF-1α, HIF-2α, or HIF-3α) and HIF-1ß (Hirota, 2020). HIF-1α and HIF-2α have 48% similarity in sequence identity but have very different roles. HIF-1α has been shown to be more active in response to acute hypoxia (2-24 hours) whereas HIF-2α appears to be active in more chronic (28-72 hours) hypoxic exposures (Koh et la., 2011).

HIF activity is downregulated and degraded in normoxic conditions due to the hydroxylation of two proline residues by oxygen-reliant HIF-prolyl hydroxylases (PHDs) (Ivan et al., 2001; Manola et al., 2005). PHDs sense oxygen and in turn directly regulate the activation of HIFs (Gaete et al., 2021). This pathway results in the binding of von Hippel-Lindau tumour suppressor (pVHL) and the recruitment of an E3 ligase complex and ubiquitin-mediated proteasomal degradation (Burslem et al., 2017). Due to the stabilisation that *VHL* provides, the loss of this gene in patients has been linked to contributing to cancer progression and metastasis (Razorenova et al., 2011). A pathway that supports tumours in hypoxic conditions is the vascular endothelial growth factor (VEGF). When HIFs binds to the *VEGFA* gene and upregulation occurs, this pathway stimulates angiogenesis (Raja et al., 2014). *HIF* also

regulates the expression of a wide array of key genes important for hypoxia-mediated biological effects, including leptin, nitric oxide synthase, hexokinase 1 and 2, glucose transport 1 and transforming growth factors α and β (Chaturvedi et al., 2014; Masoud et al., 2015; Rankin et al., 2016; Aguilar-Cazares et al., 2019). As well as all these factors, HIF-1α has been shown to regulate the expression of WSB-1, an E3 ligase which has been shown to regulate metastasis in breast cancer (Poujade et al., 2018).

1.4 WSB-1 (WD Repeat and SOCS Box-Containing 1)

WSB-1 (WD Repeat and SOCS Box-Containing 1) is the substrate recognition part of an ECS (Elongin-Cullin-SOCS box) E3 ligase complex (Hilton et al., 1998). Where it was first discovered in chicken and defined to be a target of Shh (Sonic hedgehog) signalling pathway during embryonic development (Vasiliauskas et al., 1999; Dentice et al., 2005). The ubiquitin proteasome pathway is known for its high importance for correct cellular function (Goldberg, 2003), enlightening why this pathway has been a field of interest for recognising it in cancer development.

WSB1 has three isoforms (Figure 5). Work by Archange et al., (2008) investigated the role of all three isoforms and their relation to the progression in pancreatic cancer cells. It was discovered that inducing stress on the cells increased the expression of isoforms 1 and 2. The increased levels of expression were seen to promote cell growth and sensitised the cells to apoptosis. Whereas isoform 3 of *WSB1* under stress, reduced expression and was seen to reduce cell proliferation and enhance resistance to apoptosis. This research resulted in the understanding that induced stress on WSB-1 in the microenvironment interacts with how the isoforms express and reveals an increase in resistance to apoptosis in pancreatic cancer cells. Shichrur et al. (2014) researched the silencing effects of *WSB1* on neuroblastoma cells, specifically on *WSB1* isoform 3 and concluded that the reduction in expression of these genes reduced growth, enhanced apoptosis rate and increased sensitivity to chemotherapeutic agents. Highlighting that *WSB1* isoform 3 may be a target for therapy in neuroblastoma cancer.

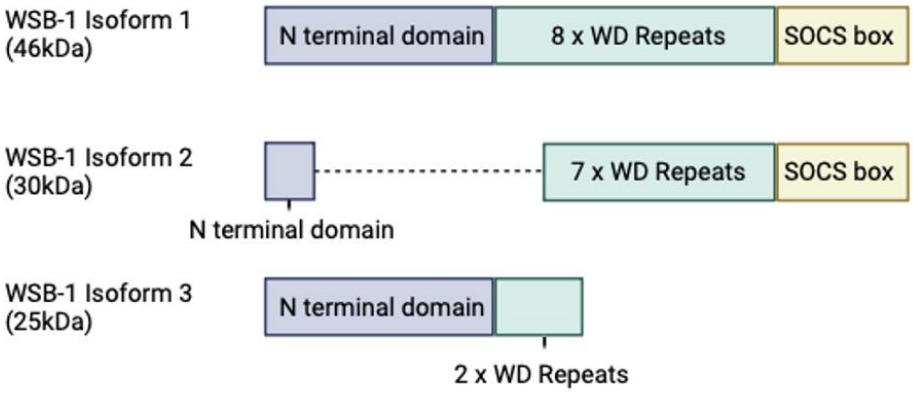


Figure 5: A diagram representing the three isoforms of WSB-1 (Figure created using BioRender).

Isoform 1 consists of three domains; N terminal domain, 8 x WD repeats and SOCS box. Isoform 2 differentiates by having a substantially shorter N-terminal domain and 7 WD repeats. Isoform 3 is shown to only have 2 WD repeats and no SOCS box domain present, resulting from a premature stop codon in the C-terminal part of the protein (Archange et al., 2008; Shichrur et al., 2014).

The formation of an E3 ubiquitin ligase regulates tumour progression and chemoresistance through ubiquitination of multiple proteins including Rho GDP-dissociation inhibitor 2 (RhoGD12), pVHL, IL-21 and homeodomain-interacting protein kinase 2 (HIPK2) and PTHrP which stimulates MMPs production, VEGF and secreted protein acidic cystein-rich (SPARC) through RUNX2 (Tong et al., 2013; Cao et al., 2015; Poujade et al., 2018) (See Figure 6).

1.4.1 WSB-1 regulation and targets

WSB-1 has been shown to have multiple signalling pathways which relate to poorer prognosis in patients suffering from cancer, figure 6 and table 5. The expression of WSB-1 was shown to increase in hypoxic conditions by Archange et al. in 2008 and later WSB-1 was found to be specifically dependent on HIF-1a (Benita et al., 2009). In 2015, in the Cao research laboratory, it was also demonstrated that in hypoxia, HIF-1a was binding to the WSB-1 transcription site -339bp, creating a feedback loop which is specifically mediated by WSB-1 degradation of pVHL (Kim et al., 2015). Reciprocally, showing that WSB-1 has regulated HIF function by targeting pVHL through WSB1 E3 ligase activity, contributing to tumour invasion, metastasis and angiogenesis (Kim et al., 2015).

HIPK2 is a tumour suppressor and works by inducing the protein p53. Protein p53 (gene *TP53*), is triggered in healthy cells through cellular stress, allowing the cell to signal and activate several transcriptional programs such as cell cycle arrest, DNA repair and apoptosis (Fernald and Kurokawa, 2013; Marei et al, 2021). Research by Tong et al. (2013) further enhanced the research gap regarding *WSB1* in tumour cells by demonstrating that the expression of *WSB1* is positively correlated with the involvement of the degradation and ubiquitination of *HIPK2*. Silencing HIF-1a in tumour cells, results in reducing the expression of *WSB1* and upregulating *HIPK2*, showing further evidence that *WSB1* interferes with *HIPK2*, downgrading etoposide-induced cell death and that *WSB1* plays a critical role in hypoxia-induced chemoresistance.

The study by Cao et al., (2015) demonstrated that hypoxia-driven *WSB1* in osteosarcoma cells promoted the proteasome-dependant degradation of *RhoGD12*, activating *Rac1* which increases the amount of F-actin and promotes the formation of lamellipodia, the degradation resulted in the increased migration movement of the tumour cells and cell motility.

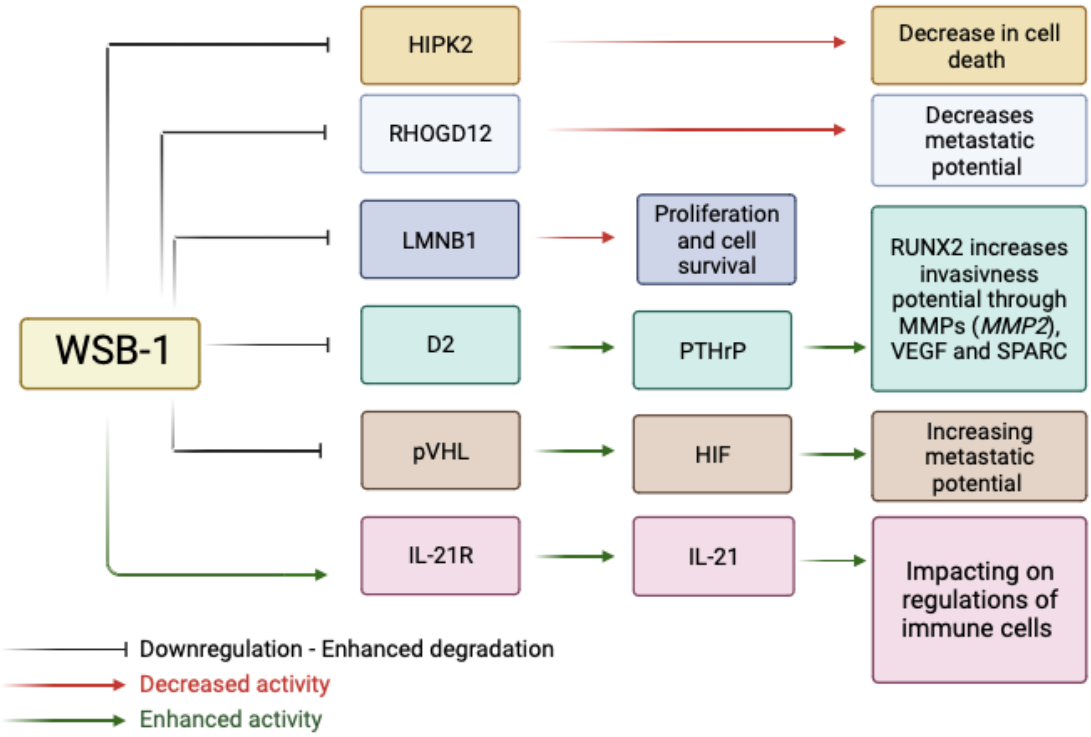


Figure 6: The multiple signalling pathways induced by WSB-1 (Figure created using BioRender).

WSB-1 interacts and downregulates VHL, upregulating HIF expression (Weng et al., 2022). Nara et al. (2011) study suggests that WSB1 has a role in the degradation of IL-21, impacting the immune system, specifically in the regulation of T-cells, B-cells, NK-cell and myeloid-cell functions. Wazir et al., (2013) found a correlation between low expression of LMNB1 in breast cancer cells to result in better patient prognosis.

Table 5: Signalling pathways induced by WSB-1

Target pathway	Role relating to cancer	Consequence of WSB1 targeting this pathway	Reference
HIPK2 (Homeodomain-interacting protein kinase 2)	HIPK2 is dependant on the p53 gene and is often found downregulated in many tumour types, often correlating with tumour progressions and chemoresistance. Although often downregulated, activation of HIPK2 can inhibit HIF1a expression, upregualting HIF1 induced angiogenesis, chemoresistance and tumor invasion.	WSB1 in DNA-damaged cells has been shown to target HIPK2 for degration via the 26 S proteasome pathway, promiting the ubiquitination and degradation of HIPK2. In hypoxic conditions, the upregulation of HIF-1, promotes WSB1, therefore incuding HIPK2 proteasomal degration and impacting the HIPK2-incudes apoptosis.	Choi et al., (2008), Conte et al., (2023)
RHOGDI2 (Rho guanosine diphosphate dissociation inhibitor 2)	RHOGDI2 can have pro or anti tumrous roles in cancer development, depending on the type of cancer and even the subtype of breast cancer. *In situ* studies have found RHOGDI2 to be highly expressed in local tumours compared to metastasised tumours. Suggesting RHOGDI2 to be a promoter in primary sites and potentially a suppressor in metastatic sites.	In hypoxic conditions such as solid tumours, the upregulation of WSB1 binds to and ubiquitylates RhoGD12, activating Rac1 which inturn stimulates cell motility and invasion.	Cao et al., (2015), Che et al., (2021), Tripathi et al., (2023)
c-Myc (Proto-oncogene c-Myc)	The gene c-Myc, encodes the transcription factor c-MYC, it is found to be highly expressed in breast cancer cells and correlates to cell cycle progression, proliferation and apoptosis. c-Myc has also been found to have high importance to the TME, regulating signalling proteins such as STAT3 and pathways including SNAIL, VEGF,IL6 AND IL-11.	Gao et al., (2022) found that WSB1 is a direct target gene of c-Myc, forming a feedforward circuit resulting in cancer development. It was discovered that WSB1 affected the β-catenin destruction complex-PPP2CA assembly and E3 ubiquitin ligase adaptor β-TRCP recruitment, which inhibited the ubiquitination of β-catenin and transactivates c-Myc.	Gao et al., (2022), Gao et al., (2023)

EZH2 (Enhancer of zeste homolog 2)	Polycomb Repressive Complex 2 (PRC2) is closely linked to tumour progression. In early stages of development, PRC2 is knowng for temporospatial gene regulation, however, dysregulation of PRC2 is associated with aberrant gene expression in the TME. EZH1/2 are core subunits of PRC2 and are highly overexpressed in the majority of cancers.	Data from Cancer Genome Atlas shows an association with WSB1 and EHZ1 and EHZ2. It is hypothesised that the connection between WSB1 and EHZ2 through the WNT signalling pathway is recruited by β-catenin proteins. Concluding that WSB1 upregulates WNT signaling hyperactivation by inducing EZH2.	Lee et al., (2022), Boldrini and Bardi, (2023)
pVHL (von Hippel–Lindau protein)	Mutations of this ligase target HIFs, upregulating this pathway resulting in tumour progression such as invasion, metastsis and angionesis.	WSB1 enhanced tumour metastasis through VHL ubiquitination and proteasomal degradation, stabilising HIFs under normoxic and hypoxic conditions. However, WSB-1 has been shown to upregulate HIF1a target genes, therefor promoting cancer invasion and metastasis.	Kim et al., (2015), Kim et al., (2017)
miR-182-5p	Through research studies, miR-182-5p has been shown to be dysregulated and either direcly or indirectly regulate tumour progression pathways such as JAKM STAT3, TGF-β, AKT and Wnt. miR-182-5p is also known to regulate MMP inhibitors. Overexpression of miR-182-5p results in poor prognosis through increased cell proliferation, invasion and cell migration.	High expression levels of circular WSB1 has been found to interact with miR-182-5p and upregulate WSB1. *In vivo* and *in vitro* models found that WSB1 is increased via the circWSB1/miR-182-5p/WSB1 axis, promoting cell proliferation and migration.	Sameti et al., (2023), Tang et al., (2024)

D2 (Type 2-iodothyronine deiodinase)	D2 has been shown to be upregulated in cancer cells through the downregulation of p53, the loss p53 elevates PTHrP production. High expression levles of D2 in tumous has been found to have a correlation with low percentage of surival in pateints.	WSB-1 enhances degration of D2 through the stimulation of Hedgehog signalling in the tibial growth plate. D2 ubiquitination via WSB-1 induces PTHrP, increasing RUNX2 and enhancing metastatic potential.	Dentice et al., (2005), Nappi et al., (2023)
IL-21R (Interleukin-21 receptor)	IL-21R is the receptor of Interleukin-21 (IL-21) is of the interleukin family, a cytokine playing an important role in cell development, differentation and proliferation, in particular, regulating T-cells, B-cells, NK cells and myeloid-cell functions. Often found to be upregulated in cancer cells, are proinflammatory and increase cytotoxicity. It is also known for activation of JAK1 and JAK3, STAT1 and STAT3.	WSB1 has been demonstrated to interact with IL-21R, enhancing the muturation of IL-21R through glycosylation. Therefor suggesting that WSB1 plays a role in IL-21R degradation and triggering inflammatory pathways.	Nara et al., (2011)
ATM (ataxia-telangiectasia mutated)	ATM has been linked to be an upstream kinase of the DNA-damage response pathway. The loss of ATM in tumour cells has been linked to comprising p53 activation and increase genomic instability.	WSB1 upregulates ATM ubiquitination in early tumorigenic progression through oncogenic stress, leading to the upregulation of WSB1, WSB1 then causing an interaction with ATM, consequently degrading ATM. Concluding in ATM degradation and the promotion of oncogene-induced senescence.	Kim et al., (2017)
NF-κb (Nuclear factor-κB)	Nuclear factors are a family of transcription factors, relied upon for a inflammatory response, they are known to have involvment in all hallmarks of cancer. The activation of NF-κb upregulates many singaling pathways including p53, STAT3, Notch and iniciates the release of IL-1.	WSB1 has been found to negatively regulate NF-κB target genes via associating with chromatin, targeting via methylated RelA (p65) for ubiquitination, terminating NF-κB-dependent transcriptions.	Taniguchi et al., (2018), Zhand et al., (2024)

Interestingly, when Che et al., (2021) overexpressed RhoGD12 in in vivo osteosarcoma cells, results found that the method reversed the spreading of WSB1-induced metastasis by reversing the expression of downstream F-actin. Additional studies by Kim et al. (2015) discovered that in osteosarcoma and epithelial cells, RNAi silencing of *WSB1* could be used for therapeutic target treatment to improve the survival of patients with metastatic diseases. Through Kim and colleagues, the investigation of *WSB1* expression levels in cancer types such as lung adenocarcinoma, melanoma, prostate cancer, and bladder cancer, it was found that higher levels of WSB-1 were more prevalent in metastatic sites than primary tumour sites (Kim et al., 2015).

1.4.2 WSB-1 and metastatic spread

WSB-1 is linked with tumour progression and spread in various tumour types, including breast, salivary carcinoma, pancreatic and urinary bladder (Rhodes and Chinnaiyan, 2005; Archange et al., 2008; Kim et al., 2015; Poujade et al., 2018). Many research studies have demonstrated that the expression of *WSB1* in tumours has led to progression and metastatic potential. Cao et al. (2015) observed the activity of *WSB1* in patients with osteosarcoma, it was revealed that *WSB1* promotes the degradation of *RhoGD12*, activating Rac1, and upregulating ROS, this degradation leads to increased migration and motility. Shichrur et al., (2014) studied the role of *WSB1* in neuroblastoma. They found that in six tumour samples, all were positive for evidence of expression of *WSB1*, isoform 3 at 26-fold higher than isoform 1 and 2. Positively, silencing all three isoforms increased sensitivity to chemotherapeutic agents and reduced tumour growth speed, suggesting a targeted therapy for patients with neuroblastoma. Silencing *WSB1* in pancreatic cancer cells in work by Archange et al., (2008) also showed an increase in cell death by using chemotherapeutic agents.

Poujade et al., (2018) established that higher levels of *WSB1* expression in metastatic breast cancer patients showed to have higher chance of distant metastasis mortality. This study was conducted using ER- and PR- breast cancer patients, *in vitro* and *in vivo.* Using qPCR analysis, it was also discovered that *WSB1* knockdown had correlations with metalloproteinase proteins, secretion factors such as vascular endothelial growth factors and angiogenic potential. Poujade et al., (2018) also found that high expression levels of *WSB1* resulted to be a poor prognostic indicator for relapse-free survival (RFS) for ER- patients. *WSB1* was also

shown to affect angiogenic potential *in vitro*, with a WSB-1 knockdown leading to a decrease in VEGF secretion.

Furthermore, Poujade et al. (2016) found that siWSB1 led to downregulation of SPARC. SPARC is known to be a protein that plays a role in adhesion and especially in metastasis to the bone (Koblinski et al., 2005). Along with the effects on SPARC, lamin B1 (LMNB1) was also affected. WSB-1 knockdown was shown to upregulate *LMNB1* expression, concluding that higher levels of WSB-1 inhibit *LMNB1* expression which Mamoon (2021) states could worsen the prognosis for patients with breast cancer. Yang et al. (2022) studied high levels of LMNB1 expression in liver cancer and found the potential to aid in cell proliferation and metastasis by upregulating the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. The study by Poujade et al., (2018) and preliminary data by the Pires laboratory, have indicated that WSB-1 may be a significant key player in bone metastasis and breast cancer in receptor-negative type tumour cells.

1.5 Gap in the literature and hypotheses

Preliminary data from the lab indicates there may be a link between bone metastasis within breast cancer development with the regulation of WSB-1. It was specifically found that WSB-1 may have the potential to drive metastasis by upregulating MMP expression and activity, promoting epithelial-mesenchymal transition and inducing invasiveness. This study is looking to establish if *WSB1* regulates bone cancer-associated signalling and whether in patients we see a correlation in these factors in *WSB1* expression.

Therefore, the working hypotheses for this study are:

- The downregulation of WSB-1 in breast cancer bone-homing tumour cells impacts the expression of key regulators in bone metastasis.
- WSB-1 gene expression has a direct correlation with the expression of known target genes in bone metastasis in breast cancer in patient samples.

1.6 Study aims and objectives

1.6.1 Aims of the thesis

This study aims to investigate the role of WSB-1 in breast cancer in bone metastasis, by investigating the significance of the impact of downregulating WSB-1 on bone metastasis key regulators in normoxic and hypoxic conditions. Additionally, to examine the correlation in patient samples of the expression of WSB-1 and known target genes of bone metastasis in breast cancer.

1.6.2 Objectives

- To perform *in silico* bioinformatic analysis of available RNA-Sequencing datasets to identify potential pathways in bone metastasis biology in the downstream expression of WSB-1.
- Confirmation of key molecules driving bone cancer downstream of WSB-1 transcript level by qPCR, and at the protein level by ELISA and Western blotting after siRNA treatment.
- Evaluation of clinical relevance of these discoveries using *in silico* analysis of patient datasets for correlation in gene expression between *WSB1* and candidate genes.

Chapter Two: Materials and Methods

2.1 General consumables

All general chemicals were obtained from Fisher Scientific, Sigma-Aldrich, or VWR unless stated otherwise. All general plastics were obtained from Starlab, Sarstedt, or Greiner unless stated otherwise. Tissue culture plastics were obtained from Greiner and Starlab unless stated otherwise.

2.2 Cell culture

The cancer cell line utilised throughout this study was the MDA-IV cell line, a bone-homing clone derived from the MDA-MB-231 TNBC cell line, kindly gifted by Professor Pennelope Ottewell (Nutter *et al*., 2014). HS-5, a fibroblast-like cell line also used in this study, was also gifted by Professor Ottewell. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Biowest) supplemented with 10% Foetal Bovine Serum (FBS) (Biowest). The cells were passaged when confluency reached 70-80% to maintain an exponential growth state of the population and were not passaged more than 30 times. For cell passaging, the cell monolayer was first washed with 1X Phosphate-Buffered Saline (PBS) (Biowest) and incubated for five minutes in 1X trypsin-EDTA (ethylenediaminetetraacetic acid) (Biowest) at 37°C. Detached cells were resuspended in fresh cell culture media and a fraction were then transferred to a fresh flask. Cells were incubated at 37°C in a humid atmosphere with 5% CO_2 (Nuaire, USA). Throughout this study cell culture samples were tested regularly to ensure mycoplasma-free cultures.

2.2.1 Cell counting

To ensure a constant number of cells were seeded per experiment these were counted before the experimental setup. Cell suspension cell concentration was determined by counting cells using a Neubauer haemocytometer and multiplying the average number of cells per quadrant by 10,000, obtaining a cells/ml concentration for the cell suspension.

2.2.2 Cell culture samples freeze down and thawing

To guarantee a constant stock of cells, sufficient cell line stocks were frozen down and stored in liquid nitrogen at the start of the study. For this, once the cells reached 70-80% confluency in a T175 flask, these were trypsinized as noted in section 2.1. The cells were then resuspended in media and spun down for five minutes at 1000 rpm. The media was then removed, and the cell pellet was washed with 1X PBS. For the MDA-231-IV cell line 8-9 vials were prepared per T175 flask in freezing media containing 10% DMSO (dimethyl sulfoxide) in FBS. Once suspended, 1 ml of the cell solution was placed into each vial. The vials were then placed into a cryovial holder (Mr Frosty, Thermo Scientific) filled with isopropanol to the appropriate amount. The freezing container containing the cryovials were then placed into -80°C storage for 24-48 hours. Following this, the vials were then transferred to a liquid nitrogen Dewer for long term storage.

To make certain that the cells are viable for future use, thawing of one vial was performed around a week after the freezing down process. Before returning a vial from the liquid nitrogen, a T25 is prepared with warm media, the cell vials were collected from the Dewer. The cryovial was thawed quickly at 37^{o}C and the contents transferred into the pre-prepared T25 flask. The following day, if the cells were at a confluency of 70-80% they were transferred into a T75 flask or if the confluency was not reached, the DMSO containing media was changed and transferred to a T75 flask later.

2.3 Exposure to normoxic and hypoxic conditions

Normoxic conditions were achieved using a humidified atmosphere in a CO^2 incubator (RS Biotech, UK) with 5% CO^2, 20% O_2 at a temperature of 37^{o}C. A H35 Hypoxystation hypoxia chamber (Don Whitley Scientific, UK) was used to achieve hypoxic conditions. Experimental oxygen levels were set to 1% O_2 and 5% CO_2 with 75% humidity and a temperature of 37°C prior to the start of the experiment.

2.4 siRNA transfection

siRNA transfections were completed using DharmaFECT transfection reagent (Dharmacon), as per manufacturer instructions. In brief, cells were seeded into 35 mm dishes, at $2x10^5$ cells per dish. Cells were left for 24 hours to allow time for adhering. Transfection then took place, using Dharmacon, Thermo Scientific non-targeting siRNA (catalogue number: D-001210-01-20) or siRNA against WSB-1 (catalogue number: M-013015-01-0020), both diluted with sterile DEPC water at a working solution concentration of 2 µM. Two transfection mixes were prepared, A and B. A contained the dilution of siRNA and serum-free media (1:1). B contained Dharmafect transfection reagent and serum-free media (1:70), each solution was then incubated at room temperature for five minutes. Once incubation was complete, both solutions were mixed and incubated for a further period of a minimum of 20 minutes to one hour at room temperature. Complete media was then added to the transfection solution up to 2ml per dish and replaced culture media in each dish, with a final siRNA concentration of 25 nM. Cells were incubated with the transfection mix for at least 24 hours before follow-up experiments were performed.

2.5 Transcript analysis

Quantitative polymerase chain reaction (RT-qPCR) was chosen as the preferred method for transcript level analysis as it is the most sensitive method for mRNA quantification and allows the detection of rare transcripts and the observation of small variations in gene expression.

2.5.1 Total RNA extraction

RNA extraction was achieved using the Aurum total RNA kit (Bio-Rad) as per manufacturer's instructions. Using the provided lysis solutions, cells were scraped and lysed from the culture dish. The cell lysate was collected into a tube and an equal volume of 70% alcohol mixed in. This solution was then pipetted thoroughly before being transferred into the Aurum mini-column with a waste-collecting tube. Following manufactures instructions, the lysate then went through a series of low and high-stringency washes, discarding waste after each centrifugation. To remove contamination genomic DNA, DNAase I was added to the column. To elute total RNA samples, at least 50 µl of elution solution was added to the column in a capped tube and incubated for one minute, before centrifuging for two minutes. The

extracted total RNA was then quantified using a Nanodrop lite spectrophotometer (Thermo Scientific) and stored at -20°C.

2.5.2 cDNA synthesis

cDNA synthesis was completed using RevertAid H Minus First Strand cDNA synthesis kit (ThermoFisher Scientific) following the manufacturer's instructions. The RNA was diluted with DEPC water to obtain 1 ug RNA in the appropriate reaction volume. A master mix was prepared to contain Reaction buffer, dNTP mix, Oligo d(T), RNAse inhibitor, and reverse transcriptase. The master mix was then added to the RNA samples and incubated in a thermocycler (Techne) at 42°C for 60 minutes and 70°C for five minutes. All cDNA samples were stored at -20°C.

2.5.3 Real time quantitative polymerase chain reaction (qPCR)

On ice, cDNA samples prepared as noted in 2.4.2 were diluted 1:20 in DEPC water. Then, a master mix was prepared containing Quantinova PCR SYBR green reagent with ROX reference dye (Qiagen) and DEPC water and the relevant cDNA. The chosen primers (table 6) were then added to the relevant wells of a 96-well plate (Applied Biosystems, Fisher Scientific) followed by the master mix cDNA solution. Once completed, the plate was sealed with optically clear plate adhesive film (Applied Biosystems).

Table 6: List of the primers used for SYBR Green qPCR

Primer target gene	Primer sequence/ Geneglobe ID	Primer manufacturer
WSB1	QT00064127	Qiagen
ICAM1	QT00074900	Qiagen
PTHLH	QT00015393	Qiagen
MMP1	QT00014581	Qiagen
MMP9	QT00040040	Qiagen
TGFBR1	QT00014581	Qiagen
PTGS2	QT00040586	Qiagen
IL1B	QT0002138	Qiagen
IL6	F - 5' CGGGAACGAAAGAGAAGCTCTA R - 5' CGCTTGTGGAGAAGGAGTT	Sigma-Aldrich
IL11	QT00074088	Qiagen
B2M	QT00088935	Qiagen
CA9	QT00011697	Qiagen
HK2	F - 5'-TGCCACCAGACTAAACTAGACG R - 5'TGAATCCCTTGGTCCATGAGA	Sigma Aldrich

The plate was spun in a plate spinner prior to placing in a StepOne qPCR machine (Applied Biosystems), where the amplification programme noted in table 7 was run (as per manufacture instructions to the Quantinova kit).

Table 7: Cycle protocol for amplification on the StepOne qPCR machine (as per Quantinova SYRB kit)

Initial denaturation	Denaturation	Annealing/extension
95^{o}C 5 minutes	95^{o}C 10 seconds	60^{o}C 3 seconds
	Repeated 35x	

2.5.4 qPCR data analysis

The data received from the qPCR machine was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). In brief, the average Ct values of each condition (gene/sample) was first determined. The average Ct values/target gene were then normalised to the housekeeping gene (*B2M*), which determined the ΔCt value for each gene for that sample. ΔCt values per experimental condition were then normalised to the control condition to calculated $\Delta\Delta$Ct values. To determine the fold change (FC), the calculation $2^{-\Delta\Delta Ct}$ was completed.

2.6 Protein expression analysis

2.6.1. Cell lysis and protein extraction

To prepare protein lysates, cells were washed with 1 x PBS. Fresh 1X PBS was then added to the dish, and cells were scraped using a cell scraper. The cell suspension was transferred into a microcentrifuge tube, spun briefly using the microcentrifuge (Eppendorf) and the supernatant was discarded. UTB lysis buffer (9 M Urea, 75 mM tris-HCL pH 7.5, and 0.15 M beta Mercaptoethanol) was used to resuspend the pellet and generate a cell lysate. Lysates were sonicated to sheer the genomic DNA for 5 minutes (30 seconds on, 30 seconds off) using a Bioruptor sonicator (Diagenode). Samples were then centrifuged for 15 minutes at 13,000 rpm at 4°C (Eppendorf centrifuge 5430 R). Supernatant was then collected and stored at -20°C.

2.6.2 Protein quantification and sample preparation

Protein sample quantification was performed using Nanodrop lite (Thermo Fisher Scientific). Samples containing 30-50 µg were prepared using UTB buffer and sample buffer (3.3% SDS, 6 M Urea, 17 mM Tris-HCL pH 7.5, 0.07 M β-Mercaptoethanol and 0.01% Bromophenol blue). All samples were heated to 95^{o}C for 5 minutes on a Techne-30 heat block and either used immediately or frozen.

2.6.3 SDS-PAGE

Prior to Western blotting, protein samples were separated using sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). 10% acrylamide-based gels were prepared using the Bio-Rad casting stations. Table 8 shows the recipe used. To separate the protein samples, the gel running cell was placed into the holder cassette. 1 x running buffer

was created using 10x running buffer (containing 30.20 g Tris-base, 114 g glycine and 100 ml of 10% SDS in MilliQ water up to 1 litre, table 9). Geneflow molecular weight ladder was also added to the appropriate wells. The gel then ran for 30 minutes at 60V using a Bio-Rad power pack. Voltage was then increased to 100V for 60 minutes or until the samples had run to the appropriate level at the bottom of the tank. Once completed, gels were wrapped in cling film and moisturised with deionised water before storing at 4^{o}C overnight or used immediately.

Table 8: Components of running gel and stacking gels for 10% Acrylamide gels (volumes for 2x gels)

	Running/Resolving gel (Volume in ml)	Stacking gel (Volume in ml)
H_2O milliQ	6.07	6.10
1.5M tris pH 8.8	3.75	N/A
0.5M Tris pH 6.8	N/A	2.50
30% Acrylamide/0.8% bis-acrylamide	4.95	1.30
10% SDS	0.15	0.10
10% APS	0.075	0.10
TEMED	0.018	0.20

Table 9: Compositions of running and blotting buffers used for SDS-PAGE and Western blotting (volumes for 10x concentrated stocks)

Reagent	Running buffer	Blotting buffer	TBS (pH 7.4)
Tris base	30.2g	30.2g	24g
Glycine	144g	144g	-
SDS (pellets)	10g	-	-
NaCL	-	-	88g
MilliQ water	Top up to 1L	Top up to 1L	Top up to 1L

2.6.4 Western blotting

Once the protein samples had run to the correct position on the gel, gels were removed from the cassette and placed into a transfer cassette with PVDF transfer membrane (Merck), which was activated with methanol for 5 seconds. These were sandwiched between a sponge and three layers of filter paper on each side of the gel, the layers were soaked in 1x transfer buffer (diluting 10x transfer buffer, 20% methanol and MilliQ water) and air pockets were removed by rolling each layer and places in a transfer cassette. The transfer cassette was placed into the tank, along with a frozen cooling block, magnetic stirrer, and filled with 1x transfer buffer. The tank was placed onto a stirrer platform and ran for 60 minutes at 100V. Once the transfer was completed, the membrane was quickly rinsed in TBST (10x TBS (see table 8), 1% Tween 20, and milliQ water) and then placed in 5% skimmed milk in TSBT to prevent non-specific binding of antibodies and onto a rocker see-saw (Stuart) for 60 minutes to block.

Once the blocking was complete, membranes were rinsed in 1% TBST and then placed into the antibody of choice (Table 10) diluted in 1% skimmed milk and TBST. The membrane was then incubated overnight at 4^{o}C. Following overnight incubation, membranes were thoroughly washed with 1X TBST for 3x10 minutes. Next, membranes were incubated at room temperature for 1 hour in the appropriate secondary antibody, diluted to 1:2000 in 1% milk-TBST. Membranes were then thoroughly washed with 1X TBST for 3x10 minutes before imaging. To image the blots a ChemiDoc XRS+ System and Image Lab software (BioRad) were used. Membranes were briefly incubated with chemiluminescence substrate (Clarity Western ECL Substrate, BioRad) then transfer to an acetate sheet for imaging on the ChemiDoc. ImageJ software were used to perform densitometry analyses of blots (Schneider et al., 2012). Bands were quantified as relative expression of the control, with beta actin as the loading control for all blots.

Table 10: List of antibodies used for Western blotting

Antibody	Manufacturer/ catalogue number	Dilution in 1% milk	Molecular weight	Animal origin of the antibody
Anti-β-Actin	Santa Cruz/ SC- 69879	1:20,000	43	Mouse
Anti-WSB-1	Novus/ 610958	1:1000	47	Rabbit
Anti-HIF1-α	Santa Cruz/ SC - 56620	1:1000	120	Mouse
Rabbit Polyclonal anti-Mouse immunoglobulin HRP	Dako/ PO448	1:2000	n/a	Rabbit
Polyclonal goat anti-Rabbit immunoglobulin HRP	Dako/ PO161	1:2000	n/a	Goat

2.7 ELISA (enzyme-linked immunosorbent assay)

2.7.1 Collection of conditioned media

Conditioned media from MDA-IV cells treated with siWSB-1 or non-targeting siRNA in both normoxia and hypoxic conditions was collected prior to total RNA extraction. The conditioned media was collected in 2ml microcentrifuge tubes, to discard debris and floating cells the tubes was spun down for 3 minutes at 1500rpm at 4◦C. The supernatant was then transferred to a fresh tube, leaving the cell debris behind, and stored at -20°C until use.

2.7.2 IL-1β (Interleukin 1β) and IL-6 (Interleukin 6) ELISA

To calculate protein secretions of IL-1β and IL-6 in conditioned media an ELISA was used, using the manufacturers' provided methodology (LEGEND MAX). Collected conditioned media was used, see section 2.6.1, of siNT and siWSB1 (normoxic and hypoxic) samples of the cultured cell line MDA-IV and added to the wells of the ELISA plate. Immobilised monoclonal antibodies specific to IL-1β and IL-6 at the bottom of the well caused antigens that were present in the conditioned media to bind. The wells were washed to remove unbound substances before

the specific enzyme-linked polyclonal antibody was added. Again, to remove any unbound antibody-enzyme reagent the well was washed. A substrate solution was then applied which developed colour in accordance with the antibody binding. To measure the concentration of the wells FLUOstar Omega plate reader (BMG Labtech) was used, set to a wavelength of 450 nm.

2.8 Co-culture

MDA-IV cells were seeded on day one into 35 mm dishes, at $1x10^5$ cells per dish and transfected on day two as per section 2.3. On day three media was removed and discarded, and the dish was washed with 1 ml PBS. 2 ml of 5% FBS media was them replaced into the dishes and placed back into the incubator or hypoxia chamber as stated in section 2.2. Additionally on day three, HS-5 cells were seeded at $2x10^5$ cells per dish in an eight well plate. On day four the conditioned media was removed from the transfected MDA-IV cells, spun down so unwanted debris would form a pellet at the bottom, 500ul of media without debris was placed onto each of the wells containing HS-5 cells. MDA-IV cells were discarded of on day four. Day six, eight, ten and twelve cells was taken out of the incubator and hypoxia chamber, the appropriate well was washed with PBS, trypsinised and resuspended in 400 ul of 5% media was added back to the well, pipetting to make sure no cells were left attached. Viable and non-viable cells were counted using the Neubauer haemocytometer as described in section 2.1.1. Trypan blue was used at a ratio of 1:1 with the cell solution when counting to ensure that calculations of viable cells vs non-viable cells could be made. Images were taken using the Olympus IX71Inverted Fluorescence Microscope on day two, four, six and eight to record cell morphology.

2.9 Patient dataset analysis

This study used the software cBioportal to perform the patient dataset analysis. The datasets utilised was Metabric (Nature 2012 and Nat Commun 2016) and Pancancer (The Cancer Genome Atlas and Pan-Cancer Atlas). Metabric (Nature 2012 & Nat Commun 2016) had 1980 total samples and Pancancer had a range from 1071 to 1084 samples. This method was performed to analyse gene expression relationships in patient samples in relation to the expression of WSB1. The mRNA expression z-scores relative to all samples (log microarray) was downloaded on 24/11/2022 for further analysis on Microsoft excel and GraphPad Prism.

The data was normalised to the median expression and transformed to log10 for the purpose of graph plotting.

2.10 Statistical analysis

All experiments were repeated independently at least three times to produce biological replicates unless stated otherwise, see relevant Figure legends for details of technical (experimental) and biological (independent) replication. Statistical significance was determined by an unpaired t-test if one variable was present, chosen due to testing independent groups. The choice of two-way ANOVA for multiple variables was also used followed by Kruskal-Wallis. Statistics for patient correlation analysis data were performed by the cBioportal, details in section 2.8. The standard deviations (SD) or standard error means (SEM) were calculated and stated on the graphs as error bars and described in the figure legends.

Graphpad Prism version 9.4.1 (July 18, 2024) was used for all statistical analyses.

Chapter Three: Results

As noted in Chapter 1, it has been previously shown that WSB-1 regulates metastatic potential in breast cancer (Poujade, 2016), and WSB-1 has a well-established role in bone development in normal physiology (Dentice, 2005). Therefore, this study aims to investigate the role of WSB-1 in breast cancer in bone metastasis. Investigating the significance of the impact of downregulating WSB-1 on key bone metastasis regulators in normoxic and hypoxic conditions. Experimental approaches include performing *In silico* bioinformatic analysis of available RNA-Sequencing datasets, gene level validation by qPCR and protein level analysis by ELISA and western blotting. This will be followed by utilising *in silico* analysis of patient datasets to evaluate the clinical relevance of the *in vitro* discoveries.

3.1 Identification of bone metastasis-relevant genes modulated by WSB-1

It was first necessary to evaluate if WSB-1 affected the expression of bone metastasis relevant factors. Therefore, the aim of this section was to identify potential candidate genes downstream of WSB-1 that are linked to bone metastasis.

Firstly, key factors relevant to breast cancer bone metastasis were selected through performing literature searches. Once the target genes to be evaluated were identified, an RNA-sequencing (RNA-seq) dataset comparing non depleted to WSB-1 depleted cells in hypoxia conditions was used, originally generated by a former PhD student in the Pires laboratory (Poujade, 2016). Specific conditions for the RNA-seq analysis were MDA-MB-231 cells transfected with either non-targeting (siNT) or WSB-1 targeting (siWSB1) siRNAs and exposed to hypoxia for 24 hours. A list of differentially expressed genes (DEGs) between siNT and siWSB-1 conditions was analysed for the presence of the breast cancer bone metastasis factors as being significantly differentially expressed when WSB-1 was depleted. Only relevant DEGs with fold changes 1.5 and higher (upregulated) or 0.6 and lower (downregulation) for WSB-1 depleted samples were selected, to allow a more stringent analysis. Table 11 displays the chosen target genes from the literature search in relation to the RNA-sequencing datasheet, a summary of the role in relation to bone metastasis in breast cancer, the fold change and significance from the RNA sequencing data. Although *PTHLH* was not significantly present in the DEG list, its expression has been previously shown to be both a key factor in bone metastasis and also as having been modulated depending on WSB-1 during bone plate development (Dentice, 2005), so it was also included in this analysis.

Table 11: A summary of the key factors identified in the literature review which are also DEGs in the RNA-seq dataset after WSB-1 depletion

Target Gene	Role in breast cancer bone metastasis	Fold Change	Significance
ICAM1	Expressed on the surface of the tumour cell to aid in intercellular adhesion and migration (Chen et al., 2022)	0.37	0.00005
IL1B	Induced osteoprotegerin secretion, awakening dormant tumour cells (Tulotta and Ottewell, 2018)	0.31	0.00315
IL6	Stimulates the production of osteoclasts (Hao et al., 2017)	0.29	0.00005
IL11	Suppressed the activity of osteoblasts. Can be secreted by the tumour cells or stimulate osteoblasts to secrete the interleukin (McCoy et al., 2013)	0.52	0.00005
MMP1	Promotes osteoprotegerin by shedding tumour-derived epidermal growth factors (Lu et al., 2009)	0.48	0.00005
MMP9	Produced by osteoclasts to assist in tumour invasion and proliferation (Liu et al., 2016)	0.19	0.0001
TGFBR1	Produced by the secretion pathway *TGF-beta* by osteoclasts to promote tumour growth (Padua and Massagué, 2009). Also accountable for providing instruction to make growth factor-beta receptor type 1 (Moore-Smith and Pasche, 2011)	0.40	0.00005
PTGS2	*PTGS2* (or *COX2*) is present in the TME when prostaglandins are derived. Produced by tumour cells and macrophage type 2 when prostaglandins are derived (Ristimäki et al., 2002)	0.34	0.00005
TNFRSF11B	Expressed by the tumour cell, binding to RANKL leading to the formation of osteoclasts (Song et al., 2022)	2.20	0.00005
PTHLH	Expressed by tumour cells and binds to osteoblast cells enhancing RANKL production and osteoclast formation (Wang et al., 2014)	0.55	0.0401

3.2 Validation of the impact of WSB-1 knockdown on the mRNA expression of key regulators of bone metastasis in breast cancer

To validate the impact of downregulating WSB-1 expression on the bone metastasis key regulators identified in section 3.1 in normoxic (20% O_2) and hypoxic conditions (1% O_2), qPCR analysis was performed. siRNA transfection with either siNT or siWSB-1 was performed on the MDA-IV bone homing cell line (derived from the parental MDA-MB-231) 24 hours before being incubated in the noted oxygen concentrations. Once incubated for 24 hours, RNA was extracted and analysed by qPCR.

To first confirm clarification that WSB-1 knockdown was efficient, qPCR analysis was performed for *WSB1* as a target gene (Figure 7). qPCR analysis shows that the knockdown of the transfection led to approximately 99% decrease in *WSB1* expression compared to siNT. This indicates that the later observations are underpinned by an effective knockdown of WSB-1 in the MDA-IV cells. As noted in section 3.1 table 10, the chosen genes included *ICAM1, IL1B, IL6, IL11, MMP1, MMP9, TGFBR1, PTGS2, TNFRSF11B* and *PTHLH,* with *B2M* as the housekeeping gene.

As can be observed in Figure 8, *ICAM1* (siNT) expression was significantly upregulated in the hypoxic setting with siNT normoxia being used as the control. ICAM1 expression can also be seen to be significantly downregulated after WSB-1 knockdown in hypoxic conditions. With the average fold change being 0.3 (approximately 70% decrease) compared to the control of siNT hypoxia.

Within the interleukin genes, three genes of interest were selected (*IL1B*, *IL6*, and *IL11*) as being DEGs significantly downregulated in the RNA-seq dataset. However, during qPCR validation, *IL6* and *IL11* expression was not significantly changed relative to the control in either normoxic or hypoxic conditions (Figure 9A-F). Interestingly, *IL1B* expression was shown to be significantly decreased after WSB-1 knockdown in normoxia conditions and highly significantly decreased in hypoxic conditions (Figure 9 A and B). In the normoxic conditions, *IL1B* was shown to be downregulated by an average FC of 0.29, in the hypoxic samples, downregulation was an average FC of 0.35.

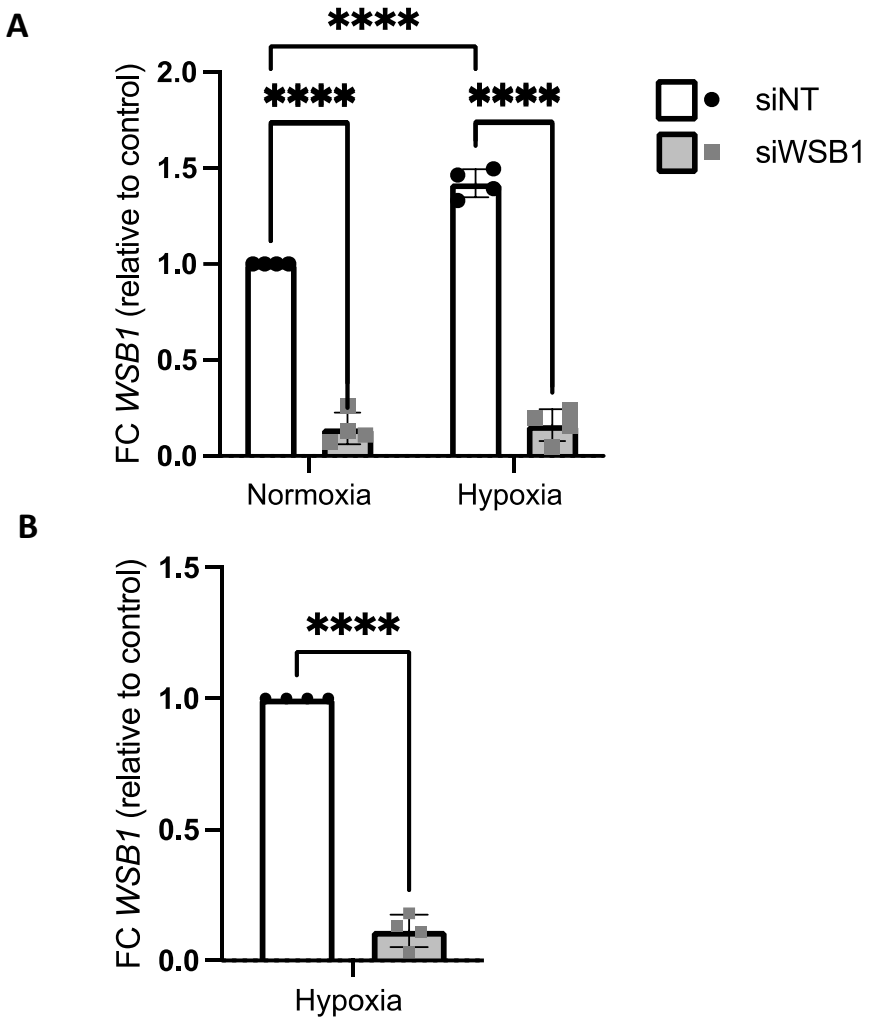


Figure 7: WSB-1 knockdown effects on *WSB1* expression on the MDA-IV cell line

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of WSB-1 (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which RNA extraction and cDNA synthesis took place. *WSB1* mRNA expression was analysed using qPCR, with *B2M* as a housekeeping gene. Histograms represent average expression of *WSB1* relative to siNT control for Normoxic (A) and Hypoxic (B) conditions for n=4 independent experiments. Error bars represent the standard deviation of the FC values. Statistical analysis was performed using the tests two-way ANOVA (A) and unpaired t-test with Welch's correction (B). **** $p<0.0001$.

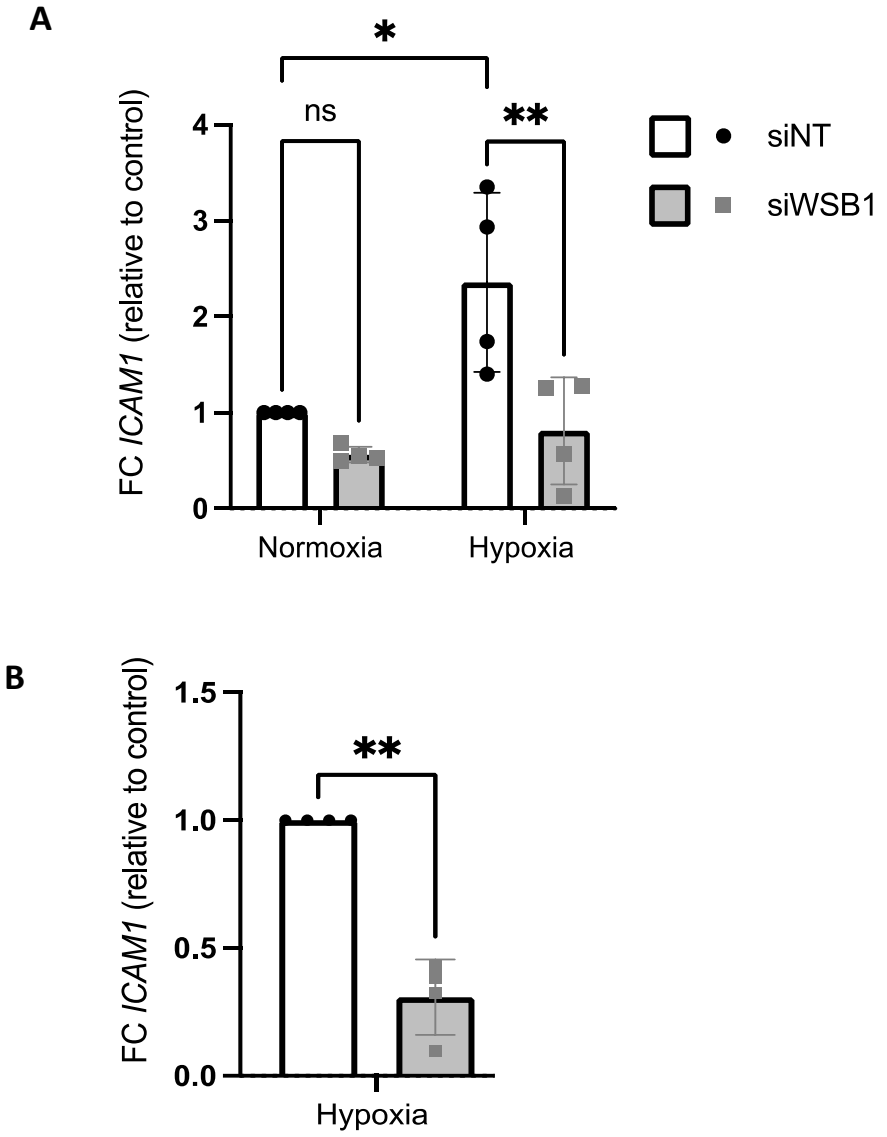


Figure 8: WSB-1 knockdown effects on *ICAM1* expression on the MDA-IV cell line

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of *WSB1* using (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which RNA extraction and cDNA synthesis took place. *ICAM1* mRNA expression was analysed using qPCR, with *B2M* used as a housekeeping gene. Histograms represent average expression of *ICAM1* relative to siNT control for Normoxic (A) and Hypoxic (B) conditions for n=4 independent experiments. Error bars represent the standard deviation of the FC values. Statistical analysis was performed using the tests two-way ANOVA (A) and unpaired t-test with Welch's correction (B). *p<0.05, ** p<0.01.

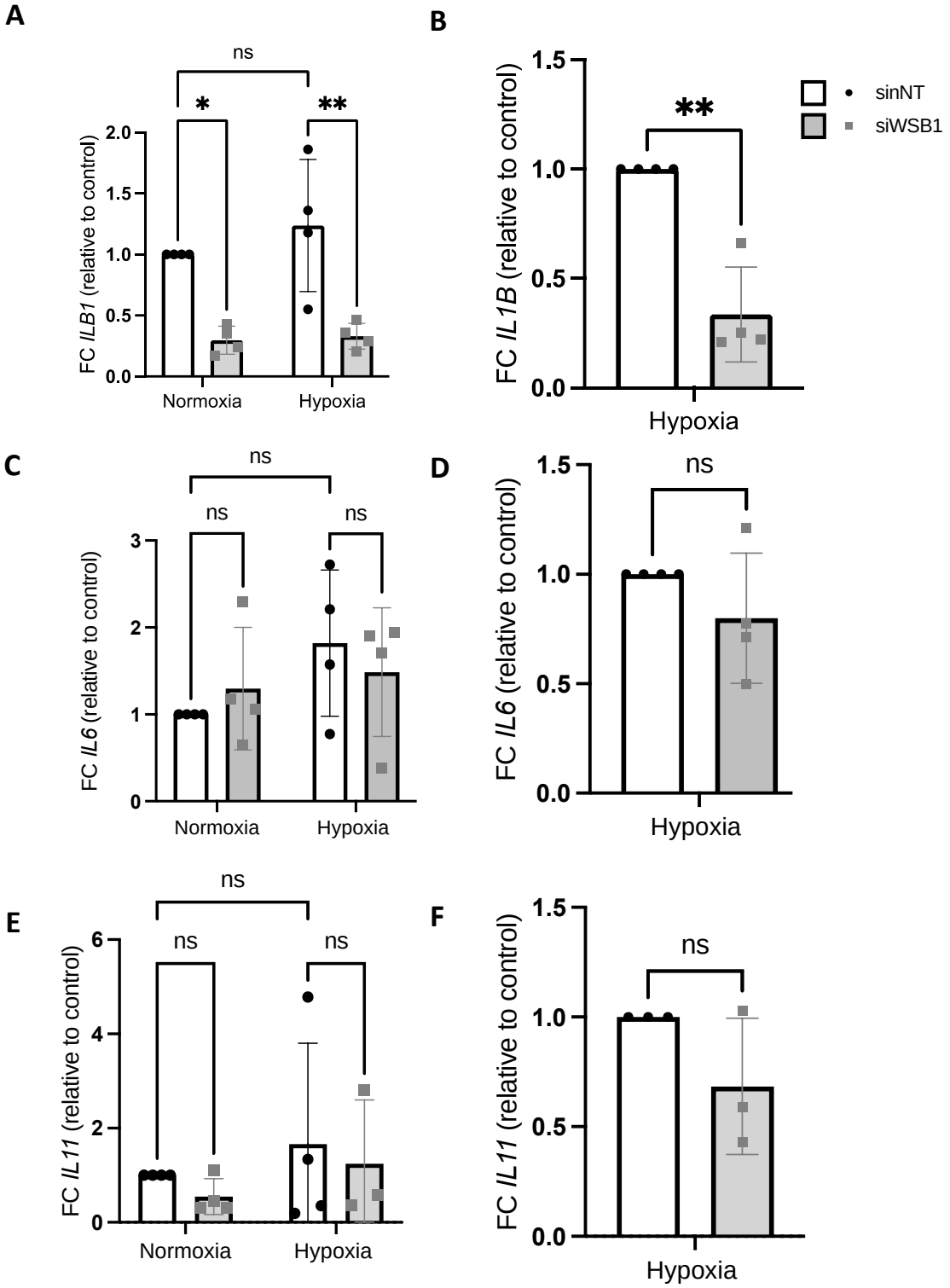


Figure 9: WSB-1 knockdown effects on *IL1B, IL6* and *IL11* expression on the MDA-IV cell line

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of WSB1 (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which RNA extraction and cDNA synthesis took place. *IL1B, IL6* and *IL11* mRNA expression was analysed using qPCR, with *B2M* as a housekeeping gene. Histograms represent average expression of IL1B (A), *IL6* (C) and *IL11* (E) relative to siNT control for normoxic and hypoxic (B, D and F) conditions for n=4 independent experiments. Error bars represent the standard deviation of the FC values. Statistical analysis was performed using the tests two-way ANOVA (A, C, E) and unpaired t-test with Welch's correction (B, D, F). ns = not significant, ** p<0.01.

MMP1 and *MMP9* were the chosen matrix metalloproteinases to be evaluated after WSB-1 depletion. Firstly, *MMP1* was found to be significantly affected by hypoxic conditions but not WSB-1 knockdown with siNT normoxia as the control. However, *MMP1* can be observed to be highly significantly downregulated in hypoxia with siNT hypoxia being used as the control, with an average fold change of 0.33 (figure 10A). Regarding the results from the unpaired t-test in relation to *MMP9*, in the dataset using siNT normoxia as the control, no results wee significant, whereas in hypoxic conditions the observed changes were significant, with a fold change of 0.4 (60% dowregulation) (Figure 10B).

There was no significant difference observed for *TGFBR1* in the normoxic conditions however within the hypoxic dataset there was a change being highly statistically significant with a downregulated expression of 75% and an average fold change of 0.37 (Figure 11).

The impact of WSB-1 downregulation on the expression of *PTGS2* when siNT normoxia is used as the control shows no significant difference in either normoxic or hypoxic settings. However, changes in hypoxic conditions were highly significant (P=0.0034), with a fold change of 0.24 (downregulation of 76%).

During the literature review*, TNFRSF11B* was shown to be of high importance in regard to bone metastasis, hypothesised to be upregulated after WSB-1 knockdown and showing to have high importance in the RANKL signalling pathway and osteoclast formation. However, the qPCR results from this study indicate no significance when WSB1 was downregulated (Figure 13).

As noted earlier, although *PTHLH* was not found to be a DEG in the RNA-seq analysis, literature found strong links between *PTHLH* and bone metastasis and between WSB-1 and *PTHLH* expression. Our data show that *PTHLH* expression was significantly downregulated in hypoxic (fold change 0.37 – 63% downregulation) conditions when *WSB1* was downregulated (Figure 14) but no significant different using siNT normoxia as the control.

The key findings from the qPCR validation on the MDA-IV bone homing cell line are that when WSB-1 is downregulated, bone metastasis factor genes including *ICAM1, IL1B, MM1, MMP9, TGFBR1, PTGS2,* and *PTHLH* are significantly downregulated. Some of these candidates will next be further validated at the protein level, focusing on interleukins IL1β and IL6, using the western blotting for IL1β levels in whole cell lysates and ELISA for IL1β and IL6 presence in conditioned media.

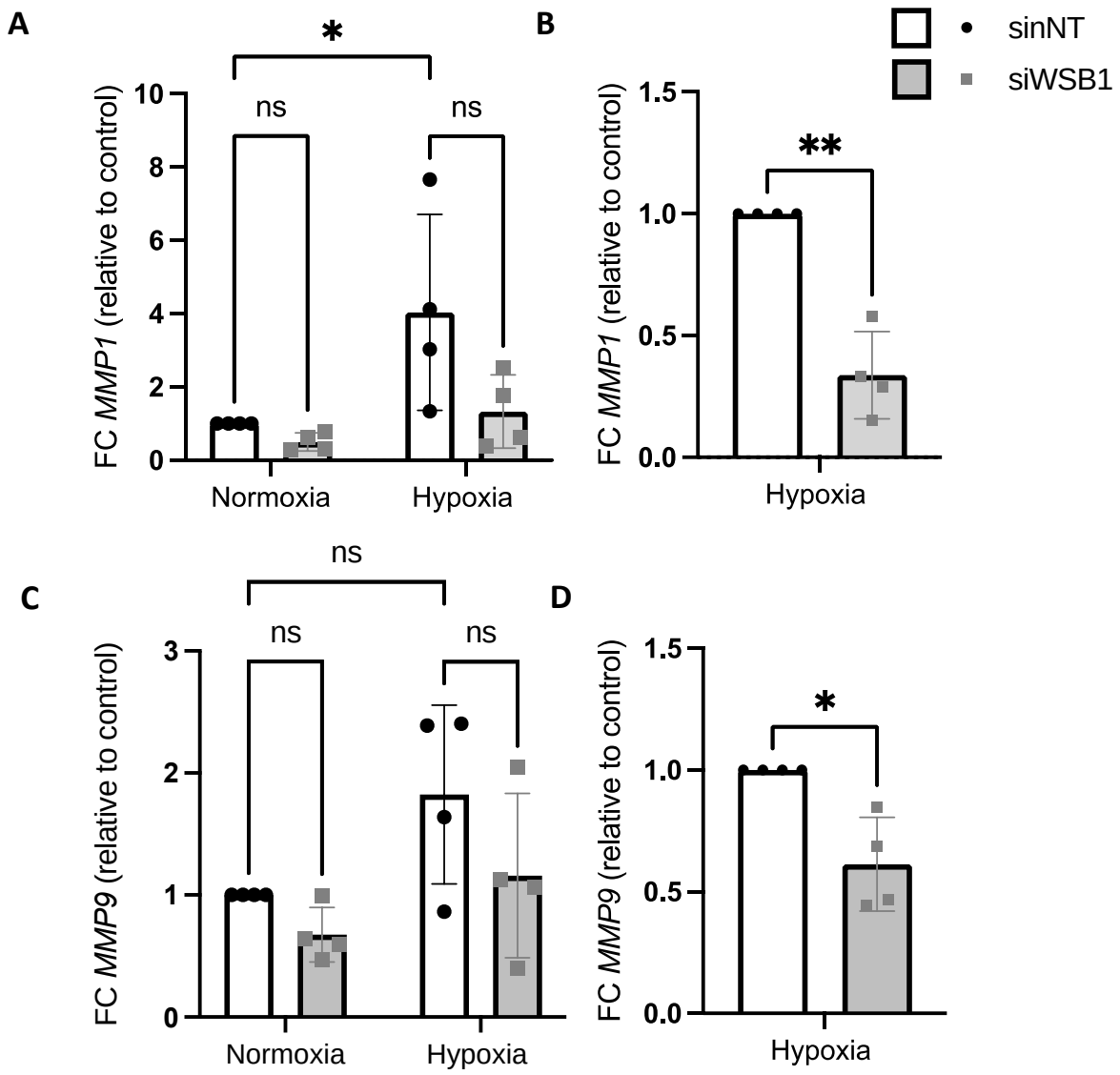


Figure 70: WSB-1 knockdown effects on *MMP1 and MMP9* expression on the MDA-IV cell line

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of *WSB1* (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which RNA extraction and cDNA synthesis occurred. *MMP1* and *MMP9* mRNA expression was analysed using qPCR, with *B2M* as a housekeeping gene. Histograms represent an average expression of *MMP1* and *MMP9* relative to siNT control for Normoxic (A and C) and Hypoxic (B and D) conditions for n=4 independent experiments. Error bars represent the standard deviation of the FC values. Statistical analysis was performed using the tests two-way ANOVA (A, C) and unpaired t-test with Welch's correction (B, D). ns = not significant, *p<0.05, ** p<0.01.

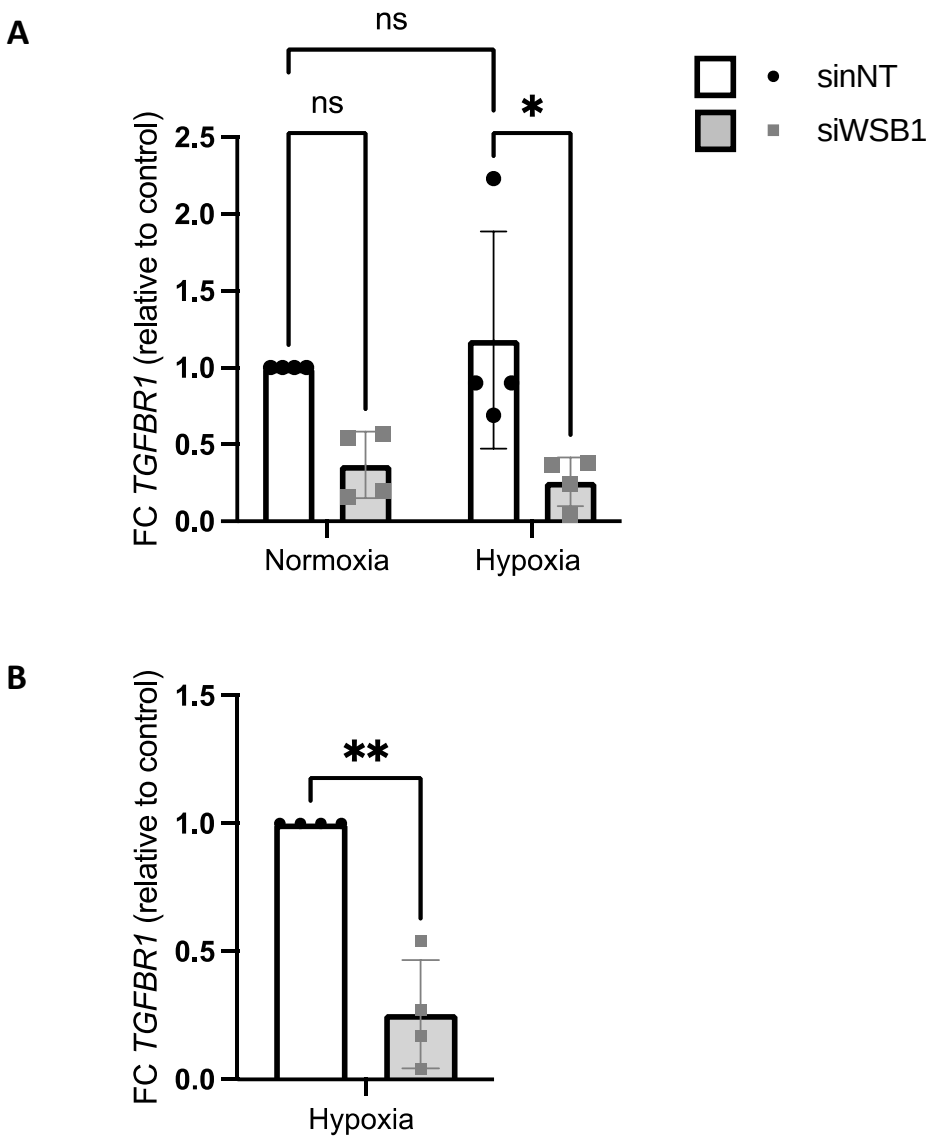


Figure 11: WSB-1 knockdown effects on *TGFBR1* expression on the MDA-IV cell line

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of WSB1 (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which RNA extraction and cDNA synthesis took place. *TGFBR1* mRNA expression was analysed using qPCR, with *B2M* as a housekeeping gene. Histograms represent average expression of *TGFBR1* relative to siNT control for Normoxic (A) and Hypoxic (B) conditions for n=4 independent experiments. Error bars represent the standard deviation of the FC values. Statistical analysis was performed using the tests two-way ANOVA (A) and unpaired t-test with Welch's correction (B). ns = not significant, *p<0.05, ** p<0.01.

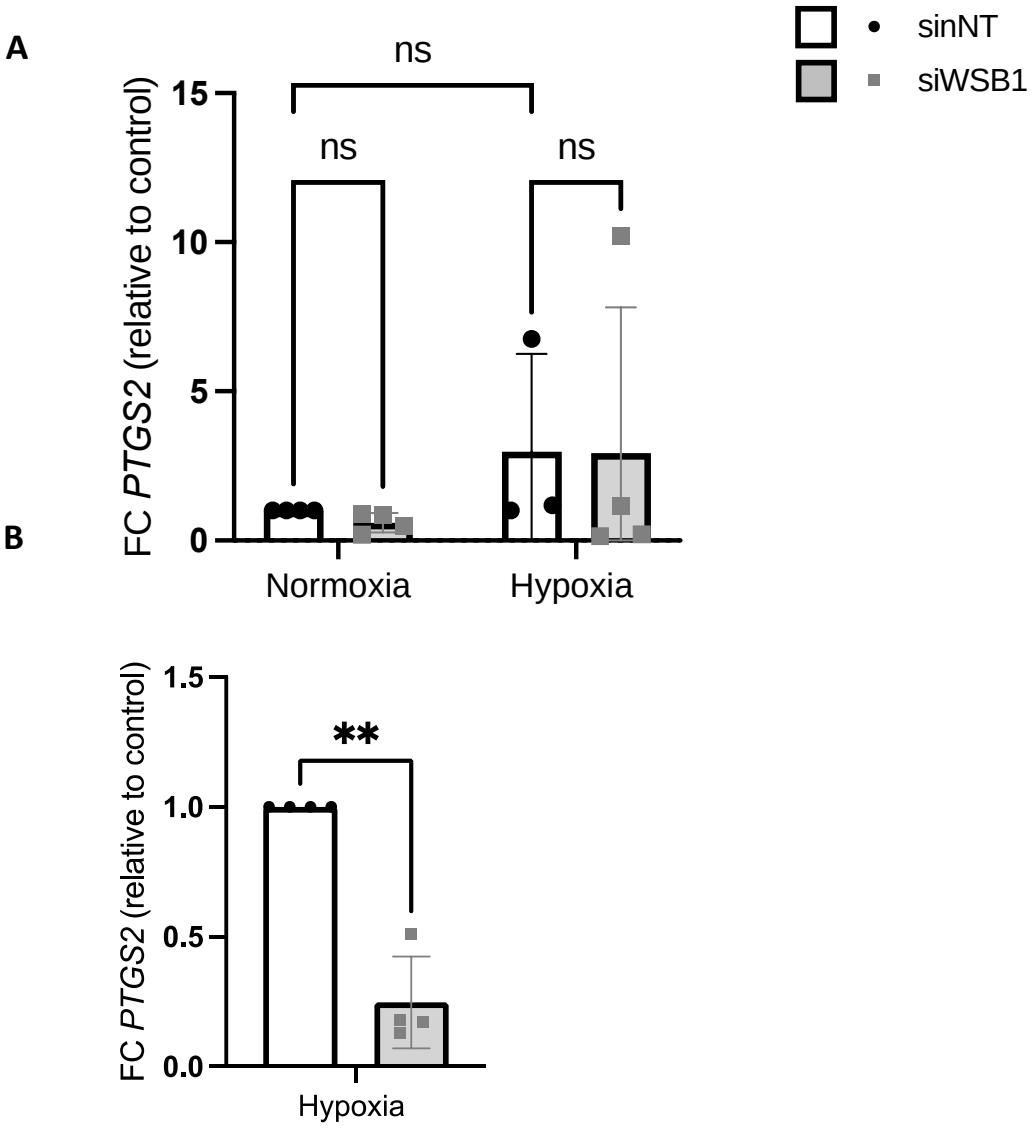


Figure 12: WSB-1 knockdown effects on *PTGS2* expression on the MDA-IV cell line

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of WSB1 (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which RNA extraction and cDNA synthesis took place. *PTGS2* mRNA expression was analysed using qPCR, with *B2M* as a housekeeping gene. Histograms represent average expression of *PTGS2* relative to siNT control for Normoxic (A) and Hypoxic (B) conditions for n=4 independent experiments. Error bars represent the standard deviation of the FC values. Statistical analysis was performed using the tests two-way ANOVA (A) and unpaired t-test with Welch's correction (B). ns = not significant, ** $p<0.01$.

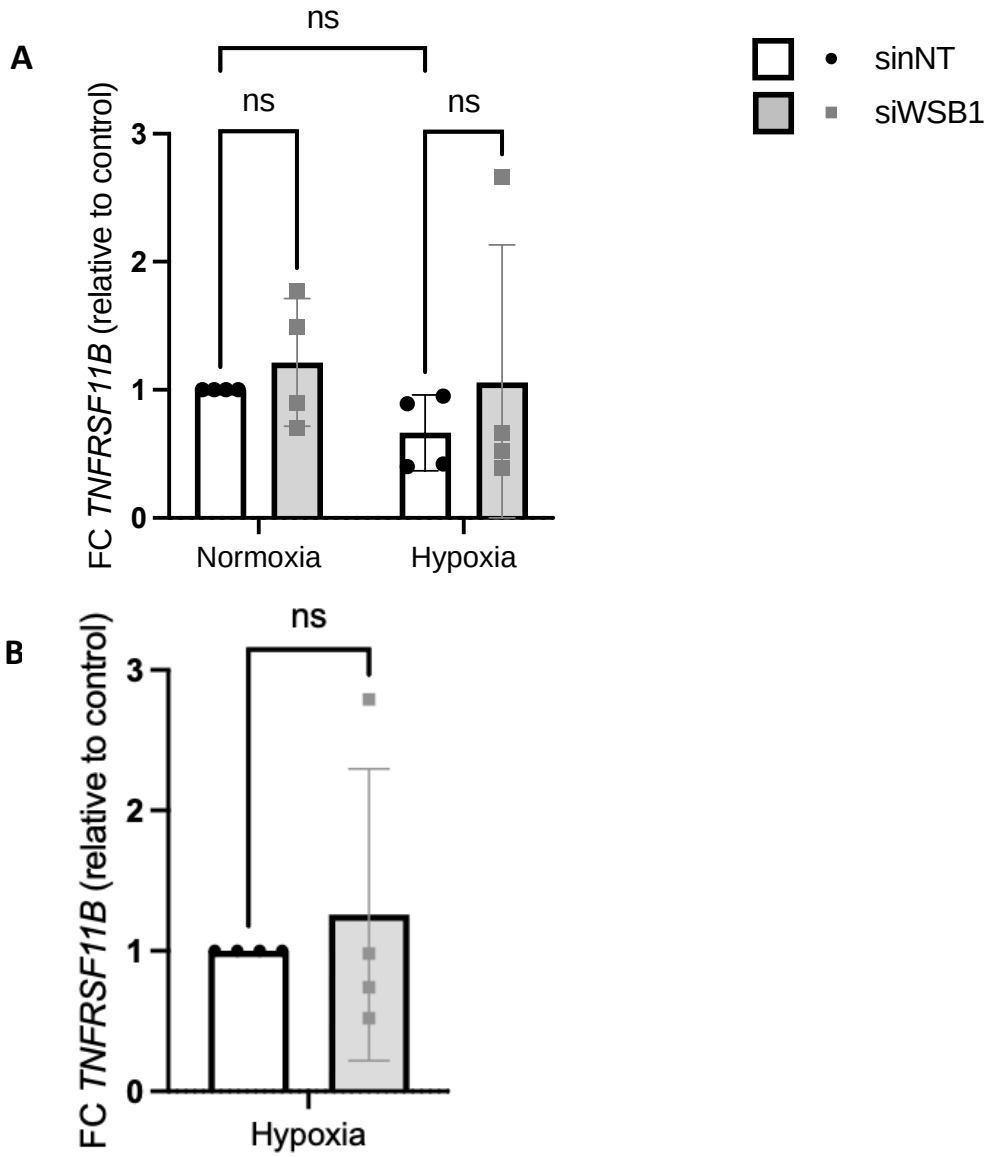


Figure 13: WSB-1 knockdown effects on *TNFRSF11B* expression on the MDA-IV cell line

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of WSB1 (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which RNA extraction and cDNA synthesis took place. *TNFRSF11B* mRNA expression was analysed using qPCR, with *B2M* as a housekeeping gene. Histograms represent average expression of *TNFRSF11B* relative to siNT control for Normoxic (A) and Hypoxic (B) conditions for n=4 independent experiments. Error bars represent the standard deviation of the FC values. Statistical analysis was performed using the tests two-way ANOVA (A) and unpaired t-test with Welch's correction (B). ns = not significant.

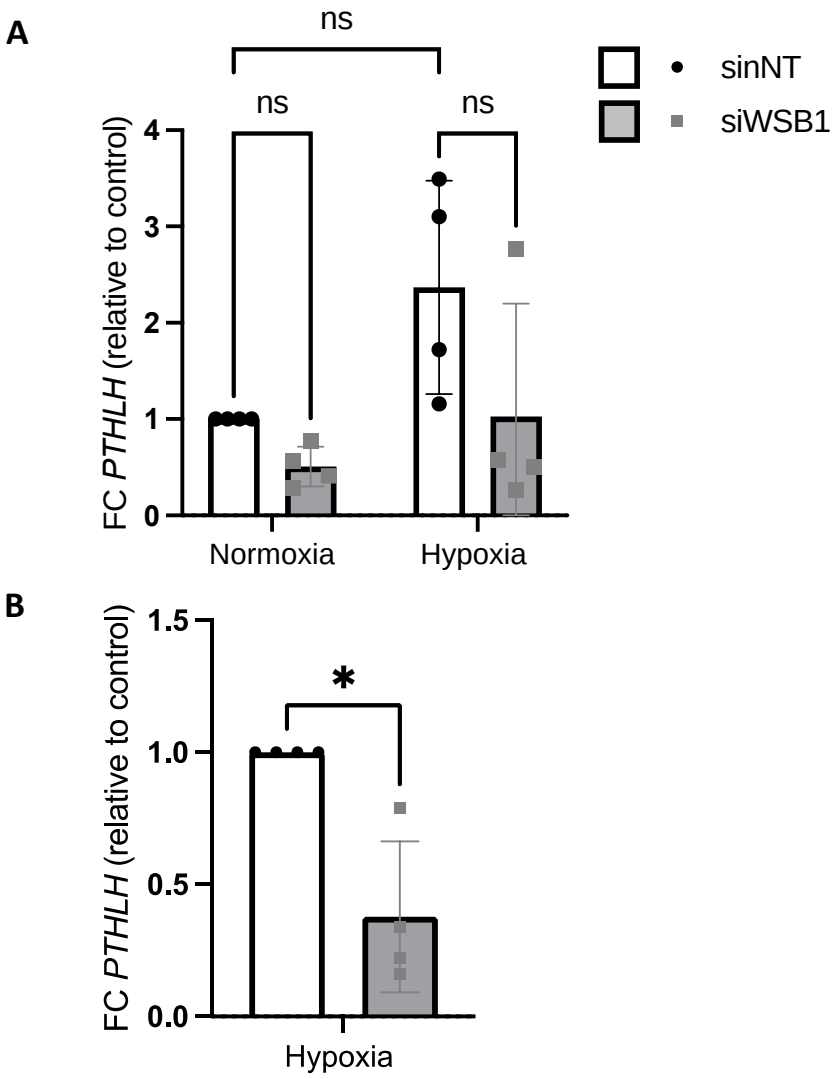


Figure 14: WSB-1 knockdown effects on *PTHLH* expression on the MDA-IV cell line

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of WSB1 (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which RNA extraction and cDNA synthesis took place. *PTHLH* mRNA expression was analysed using qPCR, with *B2M* as a housekeeping gene. Histograms represent average expression of *PTHLH* relative to siNT control for Normoxic (A) and Hypoxic (B) conditions for n=4 independent experiments. Error bars represent the standard deviation of the FC values. Statistical analysis was performed using the tests two-way ANOVA (A) and unpaired t-test with Welch's correction (B). ns = not significant *p<0.05.

3.3 Analysis of the impact of WSB-1 knockdown on IL1β protein expression

Data shown in section 3.2 in MDA-IV cells indicated that WSB-1 knockdown led to a decrease in *IL1B* transcript levels. IL1β has been shown to have relevance in metastasis to the bone in breast cancer, with high secretion levels in primary tumours indicating metastasis to the bone (Nutter et al., 2014). IL1β is reported to be secreted by the tumour cells and has a direct effect on osteoblasts in bone metastasis, specifically with osteoprotegerin (Tulotta and Ottewell, 2018). In the previous section validation through qPCR showed that IL1B appears to be downregulated when WSB-1 is knocked down. Subsequently, protein samples were extracted at a later date to investigate expression at the protein level. To confirm whether this could be seen at the protein level for IL1β two approaches were taken: analysis of IL1β in whole cell lysates using western blotting and analysis of IL1β presence in the conditioned media using ELISA. For this, as before, MDA-IV cells were transfected with either non-targeting control (siNT) and WSB1 targeting (siWSB1) siRNA and 24 hours later placed into normoxic or hypoxic conditions for a further 24 hours. After the incubation period, protein samples and conditioned media were collected. WSB-1, IL1β, and HIF-1α were analysed using western blot using β-actin as the control (Figure 15-16) and IL1β and IL6 were analysed using ELISA (Figure 17).

Regarding western blot analysis, as observed in Figure 15, HIF-1α protein levels were shown to be upregulated in hypoxic conditions (1% O_2) in all three repeats compared to normoxic conditions (20% O_2). Visually there was limited difference for HIF-1α protein levels seen between siNT and siWSB1 in both conditions, confirmed not significantly different after band quantification (Figure 16).

Western blotting did not identify a clear pattern of WSB-1 protein levels in either condition analysed. WSB-1 levels were decreased to varied degrees after knockdown, with an average fold change of 0.87 in the normoxic condition and 0.83 in the hypoxic analysis, albeit not significantly (Figure 16).

To identify if IL1β protein levels had a similar trend to mRNA expression in qPCR analysis, western blotting was first utilised. IL1β protein levels were downregulated to different degrees in normoxic and hypoxic condition after WSB-1 knockdown (Figure 16). To identify if this was significant, quantification was performed showing, although average levels are

lower, with an average fold changes of 0.48 in the normoxia and 0.92 in hypoxia, this is not significant downregulation of this protein expression when siWSB1 is present.

To examine whether IL1β and IL6 may have altered levels in condition media from cells after WSB-1 knockdown, conditioned media from MDA-IV cells treated with siNT and siWSB1 incubated in normoxic or hypoxic conditions were collected and analysed. An attempt to record the secretion of IL1β was made (data not shown), however, the secretion levels of this protein were below the detection level. Therefore, only IL-6 protein level analysis was completed with three repeats. Figure 17 shows the summary of these data, indicating that the concentration levels of this protein were not significantly altered by WSB-1 knockdown. Further discussion of this will be made in chapter four.

In summary, these results indicate that western blotting did not identify a clear indication of the knockdown of WSB-1 at the protein level using the chosen antibody in either condition. Also, the knockdown of *WSB1* did not identify a significant difference in protein levels of IL1β in connection with the controls in western blots and secretion levels in ELISA were below the detection level.

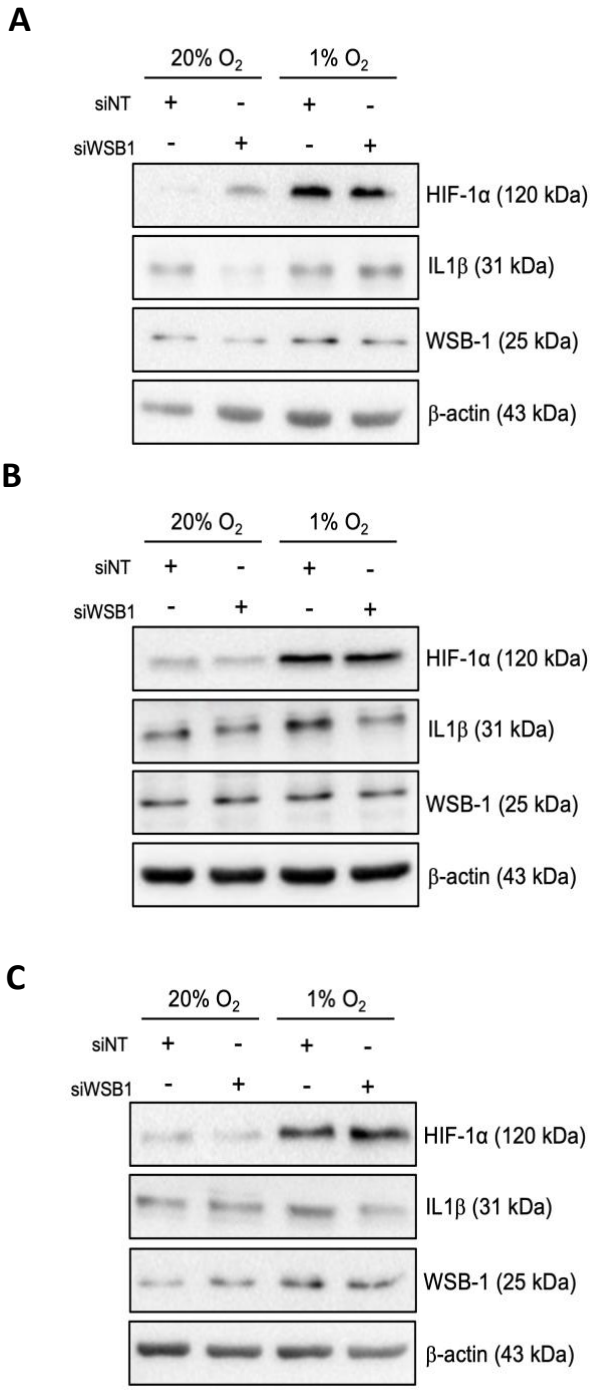


Figure 8: Evaluation of IL1β protein expression in whole cell lysates after WSB-1 knockdown

MDA-IV cells were seeded at $2x10^5$ in 35mm dishes, left to adhere for 24 hours and then transfected with either non-targeting control (siNT) and WSB1 targeting siRNA (siWSB1) and incubated for 24 hours. Cells were then exposed to either 20% or 1% O_2 for a further 24 hours. Whole cell lysates were harvested for all conditions, and samples were prepared and HIF-1α, IL1β and WSB-1 protein expression were analysed using western blotting, with 50 μg of protein was loaded per well. β-actin was used as a loading control. Panels A-C represent three independent experiments.

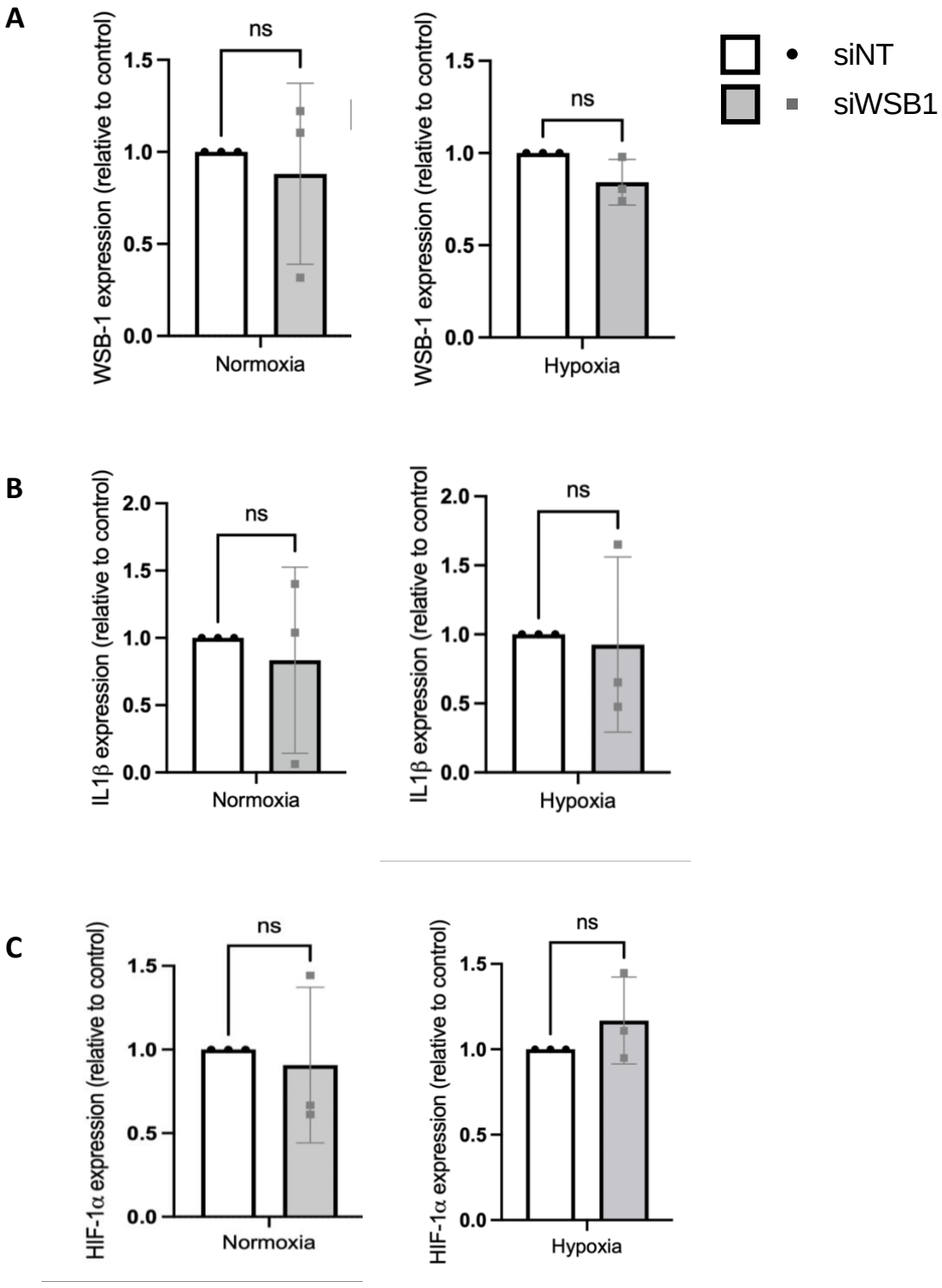


Figure 9: Densitometry analysis of protein expression in whole cell lysates

Histograms represent the average band intensity after densitometry for the western blot panels in Figure 15. Panels represent average band intensity for WSB-1 (A), IL1β (B), and HIF-1α (C). Error bars represent the standard deviation of the FC values. Statistical analysis was performed using an unpaired student t-test, ns = not significant.

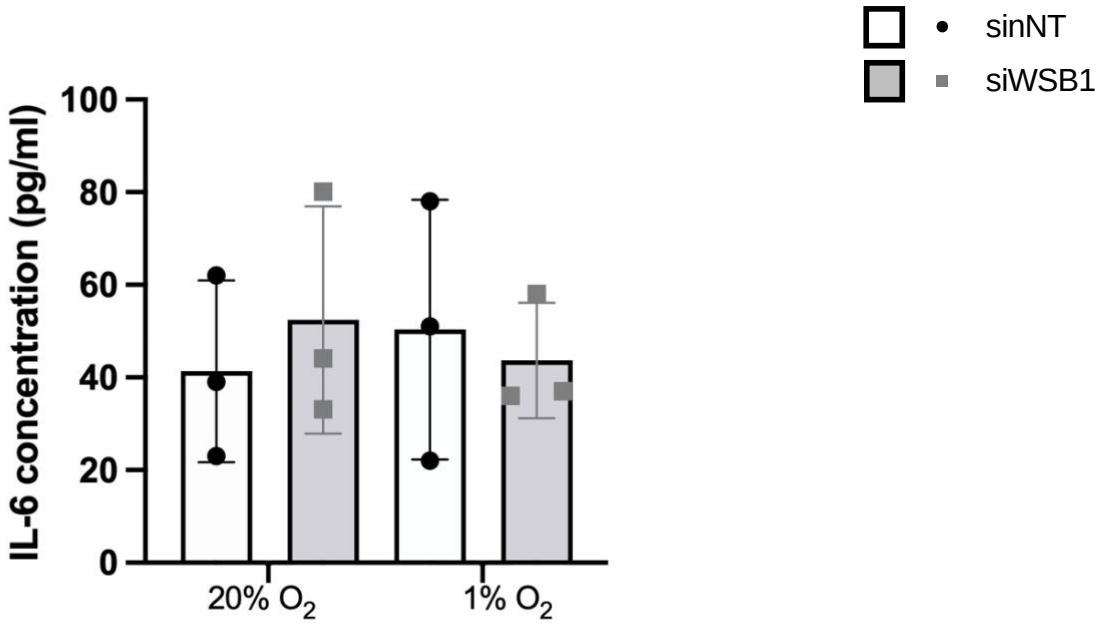


Figure 10: Impact of WSB-1 depletion on levels of IL-6 in conditioned media

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of WSB1 (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which conditioned media was collected. Analysis of the secretion of IL6 took place using an IL6 ELISA kit (section 2.6). Each point represents the concentration levels (pg/ml) detected in the conditioned media samples; Error bars represent the mean ± SEM. Statistical significance was determined using an unpaired student's t-test; no significant difference.

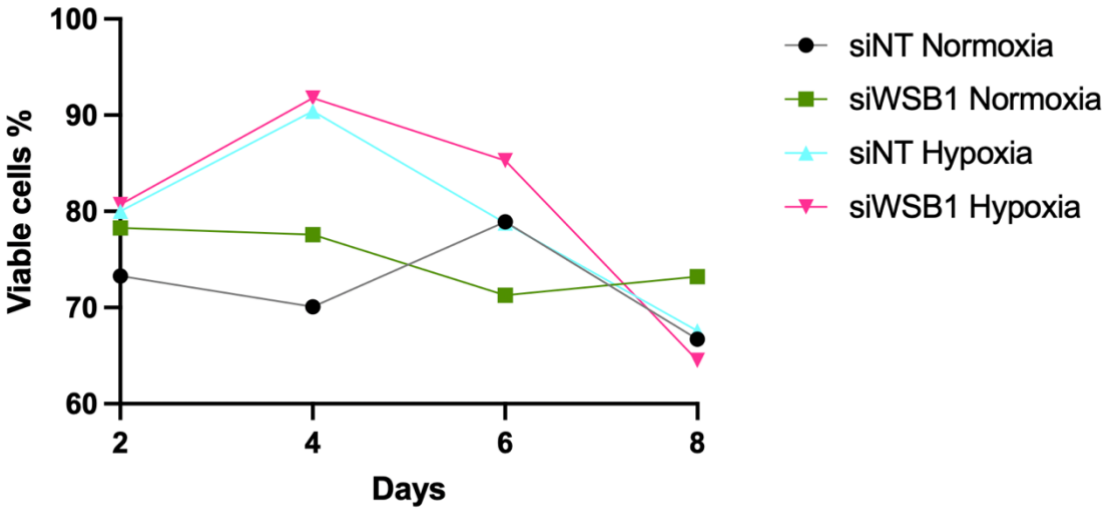
3.4 Impact of conditioned media from MDA-IV cells after WSB-1 knockdown on HS5 bone mesenchymal cells

To gain an insight into the biological impact that factors secreted from MDA-IV after WSB-1 knockdown, bone mesenchymal cells HS5 were incubated with conditioned media collected after treatment of MDA-IV cells with either siNT or siWSB1 and cell viability was analysed. Due to time constraints only one experiment was performed, with results summarised in Figure 18.

In Figure 18, when comparing the normoxia samples to the hypoxic, it is seen that on day 2 and 4 there were more viable cells in the hypoxic conditions, with normoxic remaining stable until day 4. By day 6 all of the samples apart from siNT normoxia declined in viability. Similar results were seen by day 8, with all samples declining apart from siWSB1 normoxia which inclined in percentage of viability.

In conclusion, the samples in hypoxia at the beginning of the experiment had more viable cells overall compared to cells in the normoxic conditions and by day 4 all samples apart from siNT normoxia were declining in percentage. Towards the end of the experiment (day 8), all but siWSB1 normoxia declined, siWSB1 normoxia began to increase in viability again. Overall, cells in hypoxia may have undergone proliferation quicker than normoxic but also resulted in more rapid cell death. Figure 18 shows images of the cells morphology under Hypoxic conditions treated with siWSB1 on day 2 and 4.

A



B

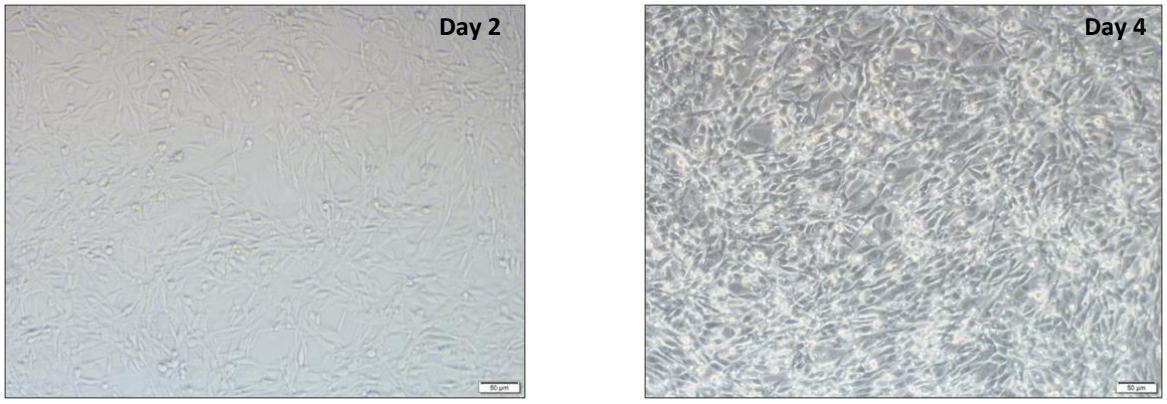


Figure 11: Impact of conditioned media from MDA-IV cells +/- WSB-1 knockdown on HS5 bone mesenchymal cells.

MDA-IV cells were seeded at 100,000 cell/well 24 hours before transfection for the knockdown of WSB1 (siWSB1) and non-targeting siRNA (siNT) (see section 2.3). 24 hours post-transfection cells were incubated for a further 24 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which conditioned media was collected. 500 ul of the collected media was placed onto each of the wells containing HS5 cells which were seeded at 200,000 the previous day. Counts were taken of the viable and non-viable cells on day 2, 4, 6, and 8 after seeding (A). (B) Representative images were taken on days 2 and 4 with the Olympus IX71Inverted Fluorescence Microscope. Scale bar = 50 μm. n=1 experimental repeat.

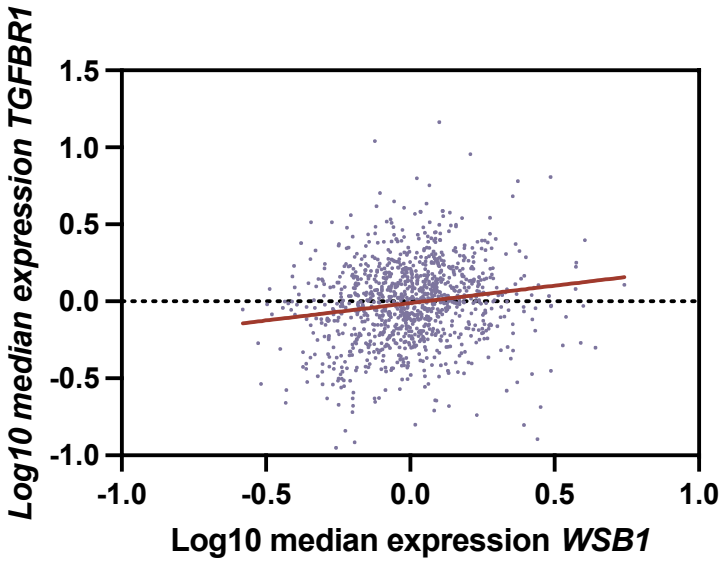
3.5 Patient data set analysis

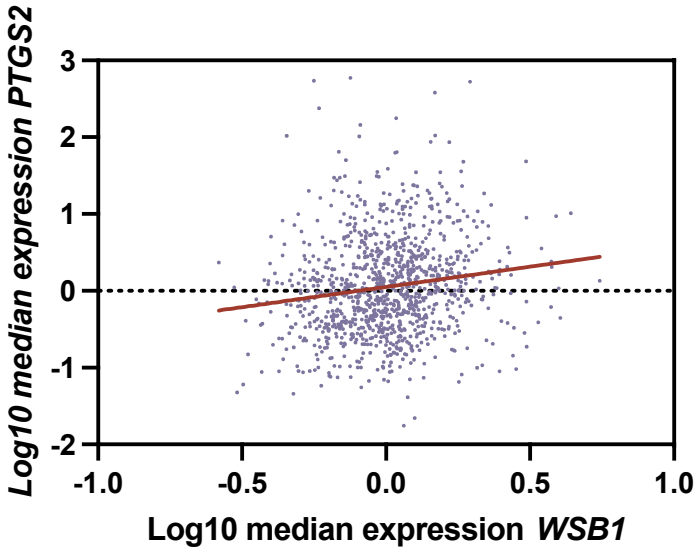
The purpose of this analysis was to take available online breast cancer patient gene expression datasets and to analyse gene expression relationships between the selected candidate genes and *WSB1* in these patient samples. Data was normalised to the median expression and transformed to log10 for statistical purposes. The data shows the correlation expression of the mRNA with the expression of *WSB1* in breast cancer samples.

Table 12 represents patient data analysis available from Pan-Cancer (The Cancer Genome Atlas and Pan-Cancer Atlas). The results found that the correlation between the mRNA expression of *TGFBR1, PTG2S, IL6 and TNFRSF11B was* highly positively and significantly related to the expression of *WSB1* in these specific breast tumour samples, graphically shown in Figure 19. The correlation between *WSB1* and *IL1B* was also seen to be very significantly positively correlated and *IL11* and *ICAM1* to be positively significant in the findings also. In contrast, both MMP1 and MMP9 (not significant) were seen to be negatively correlated, results indicate that the expression of MMP1 is very significantly negatively correlated to the expression of *WSB1* in this dataset of generic patients with breast cancer.

Table 12: The significance value of the correlation of WSB1 and observed target genes using data available by Pan-Cancer (Hoadley et al., 2018; Bhandari et al 2019)

Target Gene	Sample size	Pearson r	R squared	P value
TGFBR1	1082	0.1572	0.02470	<0.0001
PTG2S	1080	0.1532	0.02346	<0.0001
IL6	1071	0.1767	0.03123	<0.0001
TNFRSF11B	1084	0.4975	0.2475	<0.0001
IL1B	1082	0.1112	0.01237	0.0002
MMP1	1080	-0.08127	0.006604	0.0075
IL11	1081	0.07009	0.004912	0.0212
ICAM1	1084	0.06974	0.004864	0.0218
PTHLH	1084	0.04555	0.002075	0.1343
MMP9	1082	-0.0009759	9.523007	0.9744





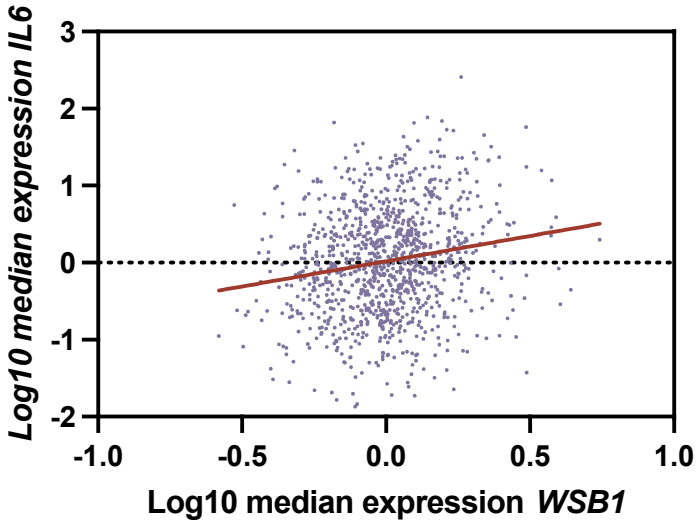


Figure 12: Correlation between the median expression of *WSB1* and *TGFBR1*, *PTGS2* or *IL6* using Pan-Cancer patient data.

Using cBioportal, data was exported from Pancancer (The Cancer Genome Atlas and Pan-Cancer Atlas). Ranging from 1071 to 1084 samples. The samples contained gene expression of breast cancer cells, including all subtypes and not specific to bone metastasis. The mRNA expression z-scores relative to all samples (log microarray) were downloaded on 24/11/2022 for further analysis on Microsoft Excel and GraphPad Prism. The data was normalised to the median expression, transformed to log10 and tested for the correlation matrix of *WSB1* and the target genes using the Pearson Correlation test. Figure shows the three most significant positively correlated genes with WSB1, *being* TGFBR1, PTGS2 *and* IL6.

Table 13 represents patient data analysis available from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC). The results found that the correlation between the mRNA expression of *PTG2S, MMP1 AND ICAM1 was* highly positively and significantly related to the expression of *WSB1* in these specific breast tumour samples, graphically shown in Figure 20. The correlation between *WSB1* and *IL6* and *IL11* was also seen to be very significantly positively correlated and *MMP9* and *TNFRSF11B* were found to be positively significant in the findings. The results showed that the expression of *TNFBR1* and *IL1B* was not significantly correlated to the expression of *WSB1* in breast cancer samples, in fact, *IL1B* was the only target gene in question which was negatively correlated in this dataset.

Table 13: The significance value of the correlation of *WSB1* and observed target genes using data available by METABRIC (Curtis et al., 2012; Pereira et al., 2016)

Target Gene	Sample size	Pearson r	R squared	P value
PTGS2	1980	0.1574	0.02476	<0.0001
MMP1	1980	0.2003	0.04011	<0.0001
ICAM1	1980	0.1010	0.01021	<0.0001
IL6	1980	0.08030	0.006448	0.0003
IL11	1980	0.08370	0.007006	0.0002
MMP9	1980	0.05029	0.04011	0.0252
TNFRSF11B	1980	0.4740	0.002246	0.0350
PTHLH	1980	0.02270	0.0005152	0.3127
TGFBR1	1980	0.02353	0.0005534	0.2954
IL1B	1980	-0.01918	0.0003679	0.3937

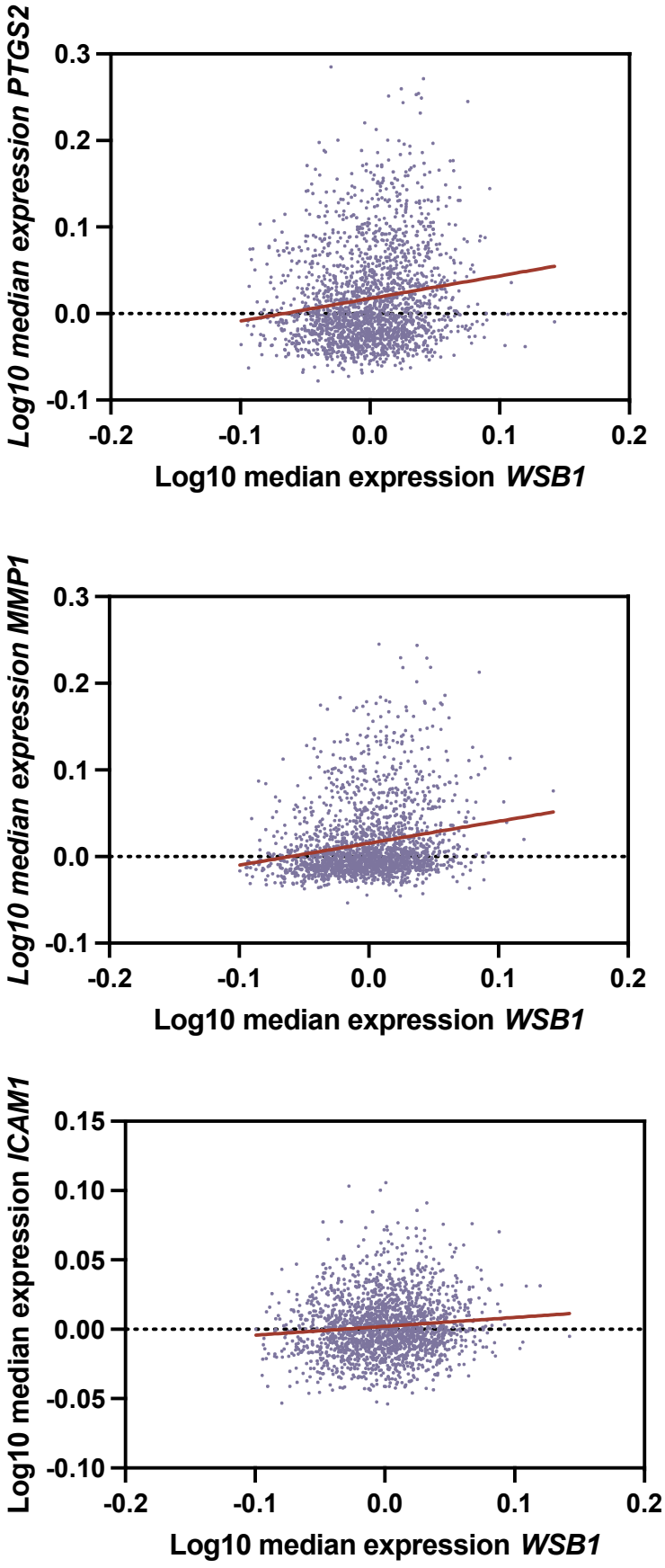


Figure 13: Correlation between the median expression of *WSB1* and *PTG2S*, *MMP1*, or *ICAM1* using METABRIC patient data.

Using cBioportal, data was exported from Metabric (Nature 2012 and Nat Commun 2016) containing 1980 samples. The samples contained gene expression of breast cancer cells, including all subtypes and not specific to bone metastasis. The mRNA expression z-scores relative to all samples (log microarray) were downloaded on 24/11/2022 for further analysis on Microsoft Excel and GraphPad Prism. The data was normalised to the median expression, transformed to log10 and tested for the correlation matrix of *WSB1* and the target genes using the Pearson Correlation test. Figure shows the three most significant positively correlated genes with *WSB1*, being *PTGS2*, *MMP1* and *ICAM1*.

In conclusion, in both datasets analysed, *PTGS2* expression was highly positively and significantly correlated to *WSB1* expression in the breast cancer samples and the gene expression of *TNFRSF11B, IL6, ILL1* and *ICAM1* were all significantly correlated to *WSB1* in both datasets, albeit a variety of significance. Although *MMP1, MMP9* and *IL1B* were significant in one dataset, they were however not correlated in the other and were in fact negatively correlated to *WSB1*.

Chapter Four: Discussion

Breast cancer is the second most common cause of female cancer-related deaths in the UK (Cancer Research UK, 2022). Unfortunately, the ability to metastasise to other sites, including the bone, is the main cause of treatment failure and results in causing more than 90% of cancer-related deaths (Wang et al., 2021). Therefore, new therapy options or useful biomarkers must be developed to treat patients diagnosed with metastatic breast cancer. WSB-1 is a protein that obtains multiple roles within tumourigenesis, and studies have shown it to be upregulated in many cancers such as breast, pancreatic, and urinary bladder (Archange et al., 2008; Tong et al., 2013; Shichrur et al., 2014; Cao et al., 2015; Kim et al., 2015). Poujade et al. (2018) found that the presence of WSB-1 downregulated MMP expression and activity and induced invasiveness, indicating that WSB-1 is an important player in breast cancer biology, specifically in metastatic spread. *WSB1* expression was also associated with a decrease in distant metastasis-free survival (DMFS) for patients with ER- and PR- breast cancer subtypes and a knockdown of WSB-1 lead to a decrease in VEGF secretion. Prior to this project, a transcriptome-wide RNA-sequencing analysis from our lab showed that WSB-1 knockdown in hypoxic conditions reduced the expression of many genes thought to have an association with breast cancer progression and bone metastasis (Poujade, 2016). WSB-1 is also known to induce PTHrP in the development of growth plates (Dentice et al., 2005), therefore, this study aimed to investigate the role of WSB-1 in relation to bone metastasis in breast cancer.

4.1 Evaluation of impact of WSB-1 downregulation on key bone metastasis factors

RNA-sequencing analysis data by Poujade (2016) was evaluated with genes that were significant to bone metastasis in breast cancer according to literature research selected for further study. To investigate the impact of WSB-1 on breast cancer cells that have homed to the bone, initial validation of candidate bone metastasis related factors downstream of WSB-1 was undertaken using WSB-1 knockdown on MDA-231-IV bone homing TNBC breast cancer cells, followed by 24 hours incubation in either normoxic (20% O_2) or hypoxic (1% O_2) conditions, after which qPCR validation took place, figure 21. To further validate these results in clinically relevant models, mRNA expression correlation was also analysed in breast cancer patient datasets. A summary of all statistical results from all gene expression analyses can be reviewed in table 14. Significant results in non-bone-homing cells regarding *ICAM1, IL1B, IL6,*

IL11, PTGS2, TNGFRF11B, MMP1, MMP9 and *TGFBR1* were seen to be validated in bone-homing cells and correlated with the patient dataset analysis. A discussion of the impact of WSB-1 modulation on specific factors is detailed below.

4.1.1 ICAM1

ICAM1 is found to be expressed on the surface of the tumour and aids in intercellular adhesion and migration in breast cancer (Chen et al., 2022). Results from the RNA-sequencing dataset show that when WSB-1 was knocked down in MDA-MB-231 cells, *ICAM1* expression was also significantly downregulated by about 60%, which was validated using qPCR in hypoxic conditions in bone-homing cells MDA-IV. Taking into consideration the results from the patient dataset analysis, *ICAM1* expression was shown to have a positive and significant correlation with *WSB1* expression in breast cancer patient samples. Together these results give a further indication that WSB-1 could have a positive effect on the expression of *ICAM1*. To further support the working hypothesis, ICAM-1 could be validated at the protein level by using an appropriate antibody and analysed through western blotting. These findings suggest further evidence that the upregulation of WSB-1 in breast cancer drives overexpression of *ICAM1* and therefore could potentially enhance tumour cell adhesion, a crucial step for metastasis. This could be analysed *in vitro* through flow adhesion assays such as spinning disks, flow chambers, or microfluidic techniques (Ahmad et al., 2015; Bahmaee et al., 2020).

Driving the expression of *ICAM1* down through inhibiting *WSB1* could have unfavourable effects in patients. A recent study by Regev et al. (2022) discovered that a deficiency in *ICAM1* in breast cancer cells developed large metastatic lesions in the lungs of mice, *ex vivo* experiments further discovered that breast cancer cells which expressed *ICAM1* were eliminated by neutrophils compared to I-CAM1-deficient breast cancer cells. Furthermore, Zhou et al. (2023) supported these findings by examining the role of *ICAM1* in TNBC and found that patients with low levels of *ICAM1* expression had shorter disease-free survival and found low levels to be related to immune escape. Conclusively, these factors should be taken into consideration when inhibiting *WSB1* expression which may result in the downregulation of *ICAM1* in future patient trials.

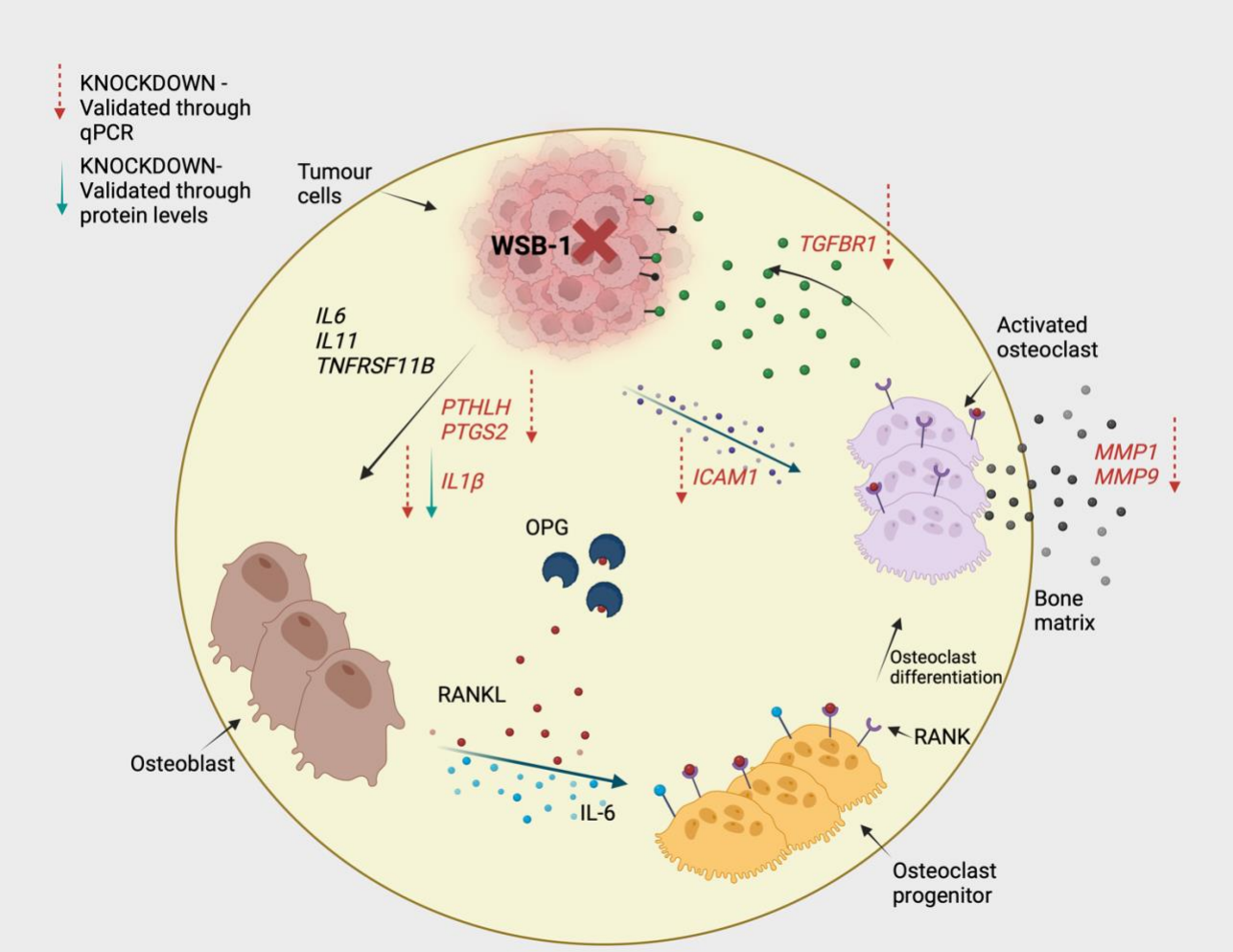


Figure 14: The impact of the knockdown of WSB-1 on MDA-231-IV cells and the cycle of breast cancer bone metastasis (Figure created using BioRender).

Abbreviations: *PTHLH*, parathyroid hormone like hormone; IL, interleukin; *TNFRSF11B*, TNF receptor superfamily member 11b; *PTGS2*, Prostaglandin-Endoperoxide Synthase 2; *TGF*β, transforming growth factor-β; *TGFBR1*, Transforming growth factor-beta receptor type 1; MMP, matrix metalloprotease; *ICAM1*, intercellular adhesion molecule 1; OPG, osteoprotegin; RANKL, receptor activator of nuclear factor kappa-B ligand; RANK, receptor activator of nuclear factor kappa-B

Table 14: Summary table comparing fold changes (FC) results between RNA-Seq dataset, qPCR validation results (2-way ANOVA), and patient dataset gene expression correlation analyses.

Target Gene	RNA-Sequencing analysis (MDA-MB-231 cells)		Average FC for qPCR analysis (MDA-IV cells)				Patient dataset analyses (Pan-Cancer)		Patient dataset analyses (METABRIC)	
	FC	p value	Normoxia	p value	Hypoxia	p value	Pearson r result	p value	Pearson r result	p value
ICAM1	0.37	0.00005	0.55	0.6773	0.3	0.0081	0.06974	0.0218	0.101	<0.0001
IL1B	0.31	0.00315	0.29	0.0189	0.33	0.0032	0.1112	0.0002	-0.01918	0.3937
IL6	0.29	0.00005	1.29	0.9199	1.48	0.8893	0.1767	<0.0001	0.08030	0.0003
IL11	0.52	0.00005	0.54	0.9566	0.93	0.9718	0.07009	0.0212	0.08370	0.0002
MMP1	0.48	0.00005	0.5	0.9584	1.32	0.0827	-0.08127	0.0075	0.2003	<0.0001
MMP9	0.19	0.0001	0.3	0.8048	1.15	0.3018	-0.0009759	0.9744	0.05029	0.0252
TGFBR1	0.40	0.00005	0.36	0.1375	0.25	0.0216	0.1572	<0.0001	0.0235	0.2954
PTGS2	0.34	0.00005	0.41	0.9972	2.87	0.9999	0.1532	<0.0001	0.1574	<0.0001
TNFRSF11B	2.20	0.00005	1.21	0.9580	1.05	0.8003	0.4975	<0.0001	0.4740	0.0350
PTHLH	0.55	0.0401	0.5	0.8273	1.02	0.1444	0.04555	0.1343	0.02270	0.3127

4.1.2 Interleukins

High expression levels of interleukins (*IL1B, IL6, IL6* and *IL11)* are known to correlate with poorer prognosis in breast cancer patients that has metastasised to the bone (Yu et al., 2009; Salamanna et al., 2019). They are found to be secreted by the breast cancer cells and cause tumour proliferation and bone destruction within the bone matrix (Salamanna et al., 2019). One example of this is that IL-6 interacts with prostaglandin E2 and COX-2, altering the RANK/RANKL/OPG ratio and inducing osteolysis (Haider et al., 2021). IL-6 has also been found to mediate the Jagged1 and Notch pathways, leading to the conclusion of participating in the vicious cycle of bone metastasis (Sethi et al., 2011; wu et al., 2019).

Results from the RNA-sequencing dataset identified *IL1B*, *IL6*, and *IL11* as significantly downregulated in MDA-MB-231 cells after WSB-1 knockdown. Interestingly, despite *IL6* and *IL11* playing key roles in the signalling pathways of bone metastasis, qPCR analysis of gene expression after WSB-1 knockdown in the bone homing cell line model did not lead to significant gene expression changes, albeit with a trend for decreased gene expression. Through literature research and the patient dataset analysis, both *IL6* and *IL11* were considered significant candidates for further investigation, having a significant positive correlation to *WSB1* expression. However, no significant difference within samples was observed when secreted IL6 levels in conditioned media were analysed.

If future work should focus on downregulating IL6 through siWSB1, caution should be taken during patient trials. Research by Loganadane et al. in 1997 found that inhibiting thrombospondin-1 results in downregulating the expression of *IL6.* This study found that the decrease in *IL6* led to increased cell density, increased metastatic potential and drug resistance. Furthermore, a study by Hailemichael et al. (2022) also indicated that blocking IL-6 expression in murine models increased $CD4^+/CD8^+$ effector T cells. Studies show that this increase in breast cancer patients could reduce survival expectancy and lead to tumour progression (Rad et al., 2015; Yang et al., 2017).

Unlike *IL6* and *IL11*, *IL1B* expression was shown to be significantly downregulated in normoxic conditions and hypoxic conditions after WSB-1 knockdown in MDA-IV cells. IL1β (*IL1B*) is a key factor in bone metastasis in breast cancer, with upregulation of *IL1B* within the bone met upregulating osteoprotegerin (OPG) secretion through the via p38 and p42/22 MAPK

signalling pathway (Tulotta and Ottewell, 2018). The upregulation *IL1B* expression was also significantly and positively correlated with *WSB1* expression in patient datasets. Protein levels of IL1β were initially evaluated through western blotting, which indicates that WSB-1 knockdown in hypoxia could have a downregulating effect on the protein levels of IL1β in two of the three repeats, although the results were not significant. Western blot experiments in this study did not result in the downregulation of WSB-1 at the protein level, due to the results it can not be ruled out that IL1β was not significantly downregulated because WSB-1 may have not been suppressed at the protein level either, further details are discussed in section 4.2, study limitations.

As previously indicated, an ELISA to detect IL1β secretion in conditioned media was attempted but the levels of secreted IL1β were too low to be detected, this is similar to what Nutter et al. (2014) observed. To overcome this, Lipopolysaccharide (LPS) could potentially be utilised, as these have been shown to increase the expression of interleukins (Kelly et al., 2015). LPS works by stimulating the immune response by interacting with the membrane receptor CD14 (Tsukamoto et al., 2018). This interaction generates cytokines such as tumour necrosis factors and IL1β and IL6 (Koh et al., 2018). Research by Zhou et al. (2022) used this method and found success. Since leaving the Pires research laboratory, a colleague has performed research using LPS as a stimulant and has been able to measure IL1β and observe a decrease in IL1β levels in conditioned media after WSB-1 depletion (Olivia Dean, unpublished). These findings further suggest that changes in WSB-1 levels in tumour cells may have a direct impact on interleukins, specifically IL1β. IL1β has been highlighted by previous research as a potential biomarker and target with higher levels indicating that metastasis chances are at a higher risk when overexpression is present (Tulotta and Ottewell, 2018; Lefley et al., 2019; Tulotta et al., 2019;). This could potentially indicate that since *IL1B* is significantly downregulated when WSB-1 levels are low, WSB-1 knockdown and/or inhibition could slow down the vicious cycle of bone metastasis, ultimately improving clinical outcomes. Overall this implies that high expression of WSB-1 could drive the overexpression of IL1β, therefore inducing osteoprotegerin secretion and having a direct impact on the vicious cycle of bone metastasis.

4.1.3 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (*MMP1, MMP2, MMP9, MMP11, MMP13*) have also been shown to have a positive correlation with tumour progression, being overexpressed in cancer cells, leading to the degradation of extracellular matrix proteins, increasing metastasis and invasion potential (Merdad et al., 2014; Kwon, 2023). *MMP1* is recognised to promote osteoprotegerin by secreting tumour-devived growth factors such as amphiregulin and TGFα (Lu et al., 2009) and *MMP9* promotes osteoclastic activity, assisting in tumour invasion and proliferation (Lui et al., 2016). Both extremely important players for bone metastasis.

Results from the RNA-sequencing data set saw that *MMP1* had a significant downregulation of 50% and that *MMP9* had a downregulated expression of 80%. Due to the RNA-seq results and the importance of their role in the literature search, further experiments were carried out through qPCR analysis in MDA-IV cells. Both *MMP1* and *MMP9* indicated that the knockdown of *WSB1* had a significant effect in hypoxic conditions, being 60% downregulated. *WSB1* and *MMP9* are known to be induced in hypoxic settings so it is interesting to see that when *WSB1* is knocked down, *MMP9* is downregulated too but with no effect in normoxic oxygen levels (Yan et al., 2016). This suggests that *WSB1* may have a direct influence on *MMP9*. Inhibiting the expression of *WSB1* could therefore, in turn, reduce *MMP9*, disrupting *RUNX2* expression and therefore may reduce the ability to form osteolytic lesions (Clézardin et al., 2021).

During this research project, the of of the key focus was on how WSB-1 affects interleukins, however, if time allowed it may have been a good opportunity to explore more in regards to matrix metalloproteinases, particularly at a protein level. Downregulation of *MMP1* and *MMP9* caused by WSB-1 knockdown could be validated through western blotting and further verified by ELISA, as seen by Stark et al (2007) and Pivetta et al. (2011). Due to the relationship known between MMP9 and proliferation, it may be of future interest to use a proliferation marker such as Ki-67 (MKI67) at the protein level through western blotting as Ki-67 is known to be highly expressed in proliferating cells of all phases of the cell cycle (G1, S, G2 AND M) but absent in resting cells (Sun and Kaufman, 2018).

4.1.4 TGF-beta signalling

TGFBR1 is expressed on the tumour cell and is the receptor that TGFβ binds to (Zhang et al., 2022). Studies have shown that the overexpression of TGFβ contributes to oncogenesis, binding and activating downstream receptor-regulated pathways such as SMADs, RAS, c-Jun N-terminal kinases (JNK) and Ras Homolog Family Member A (RHOA) (Korpal and Kang, 2010; Xu e al., 2015; Agyemang et al., 2022). Results from the RNA-sequencing dataset show that when WSB-1 was knocked down in MDA-MB-231 cells, *TGFBR1* expression was also significantly downregulated by 60%, which was validated using qPCR in both normoxic and hypoxic conditions in bone-homing MDA-IV cells. Results from the qPCR analysis indicate that knockdown of *WSB1* has a direct effect on *TGFBR1* expression, showing a downregulated expression of 75% in hypoxic conditions. A future direction of research may be in utilise *TGFBR1* inhibitors with *WSB1* knockdown and observe the effects this may have on the metastatic properties. Fico et al. (2019) studied the impact of *TGFBR1* inhibitors on breast cancer stem cells and their metastasis outcome and it was found that suppressing TGFβ signalling through *TGFBR1* inhibitors increased the number of tumour-initiating cells *in vitro*. Fico et al. (2019) findings suggest the possibility that treatment with TGFβ inhibitors might promote the likeliness of circulating tumour stem-like cells, and therefore caution must be taken when downregulating the production of *TGFBR1* in patients with breast cancer in future trials.

4.1.5 COX2/PTGS2

PTGS2 is found to be overexpressed in 40% of metastatic breast cancer, research has also revealed that upregulation of this gene may be a vital part of the formation of osteolytic bone metastasis by increasing PGE2 production (Singh et al., 2007; Li, et al., 2008). Results from the bioinformatics analysis found that *PTGS2* was significantly downregulated by nearly 70% when WSB1 was knocked down in MDA-MB-231 cells. These results and findings from literature in regard to bone metastasis lead to the expression of this gene expression being analysed through qPCR. Through qPCR analysis of MDA-IV cells, it was found that *PTGS2* was only statistically significant in the siWSB1 hypoxic samples, being 76% downregulated. This is an interesting observation due IL1β having a direct influence on the production of *PTGS2* (Lee et al., 2010). IL1β was also highly downregulated in hypoxic conditions. Alongside these

findings, *PTGS2* is also well known to be induced through HIFs so to be so significantly downregulated without the presence of *WSB1* indicated that *WSB1* does have a direct correlation with the expression of *PTGS2*, supporting the working hypothesis of this study. To conclude the findings within the bioinformatics and qPCR analysis data, the patient dataset analysis also follows this trend. Both datasets showed a positive correlation between the expression of WSB-1 and *PTGS2* (p=<0.0001).

Further investigation regarding this gene of interest would be to look into the protein expression through western blots and ELISA (Gan et al., 2016). Increased levels of *COX2* in breast cancer cells have increased bone metastasis potential, believed to be because of the alterations of the prostaglandin E_2 recruitment of Tregs (Karavitis et al., 2012). Maroni et al. (2018) studied the effects of a PTGS2 inhibitor *in vivo*, results found that inhibiting the expression reduced and delayed bone metastasis and enhanced the survival of the mice. Furthermore, Karavitis et al. (2012) studied the inhibition of *PTGS2* and concluded that work did halt the development of osteolytic bone metastasis (Karavitis et al., 2012). Taking these findings into consideration, high levels of WSB-1 in patients could be treated with a *COX2* inhibitor, potentially prolonging patient life and reducing metastatic development.

4.1.6 Osteoprotegetin/RANK (TNFRSF11B)

TNFRSF11B is strongly considered to have an impact on bone metastasis in breast cancer, *TNFRSF11B* (RANK or Osteoprotegetin) is a key regulator for osteoclast development, being expressed from the tumour cell, binding to the RANKL pathway resulting in the formation of osteoclasts (Song et al., 2022). Results from the RNA-sequencing dataset show that when WSB-1 was knocked down in MDA-MB-231 cells, TNFRSF11B was significantly unregulated. In correlation to those results, there was also no significance seen in the expression at the gene level through qPCR of treated MDA-IV cells. However, in the Pan-cancer patient data set, *TNFRSF11B* results did have a highly significant correlation with *WSB1 (*<0.0001) in all breast cancer tumour samples. Due to OPG being secreted by the tumour cells, it may be noted that in future research, an ELISA may give an insight into whether siWSB1 has an effect on these levels in the conditioned media of treated MDA-IV cells. ELISA protocols of serum OPG levels, seem to be a common and effective method to analyse TNFRSF11B activity in patients and

has been performed many times in research (Vik et al., 2015; Widschwendter et al., 2015; Kiechl et al., 2017; Rachner et al., 2019).

4.1.7 PTHLH

PTHLH encodes the production of parathyroid hormone-related protein (PTHrP). This protein is produced by the tumour cell and is known for increasing RANKL production in bone metastasis (Swami et al., 2023). Work by Dentice and colleagues in 2005 found that the decreation of PTHrP was modulated by WSB-1 in the development of tibial growth plates, with PTHrP regulating chondrocyte differentiation. This research highlighted that WSB-1 mediates a key mechanism in the PTHrP pathway in the development of skeletogenesis.

The results from the RNA-sequencing dataset show that when WSB-1 was knocked down in MDA-MB-231 cells, *PTHLH* expression was downregulated by about 45%, albeit not significant. The knockdown of *WSB1* on the expression of *PTHLH* through qPCR in MDA-IV cells was significantly downregulated in hypoxic settings by nearly 65%. This correlation between *WSB1* and *PTHLH* was however not seen with the patient dataset analysis, with neither dataset showing significance. Additional time in the laboratory would have seen a benefit in exploring *PTHLH* at the protein level. This would have been accomplished with the western blot method and an effective antibody and also through ELISA work. Alongside gathering data for the expression of *PTHLH*, it would be interesting to see if the downregulation shown through qPCR also had an effect on RANKL production. This method could be carried out in the future by detecting RANKL through an ELISA and also RANKL messenger RNA expression through the qPCR protocol. Research by Meednu et al. (2021) has shown this method to be an effective research method. Overall, a downregulation of *PTHLH* expression has been shown to slow tumour-to-bone progression and reduce bone-homing breast cancers (Swami et al., 2023).

4.2 Study limitations

Although this study did highlight that WSB-1 could have a significant effect on target genes associated with breast cancer bone metastasis there are several limitations within this study. Firstly, during the western blot experiments, the WSB1 antibody used was shown to be quite

unreliable at proving any knockdown at the protein level. Previous work in the laboratory by Poujade et al., (2018) did find a reliable link between WSB-1 knockdown of at transcript and protein level within the MDA-MB-231 cell line. Unfortunately since this work has been completed, the batch of antibodies has been discontinued and usage of other manufacturers, including the one used in this study, has proven difficult to visualise the WSB-1 isoforms through western blotting. However, due to the robust knockdown of *WSB1* at tracsript level and our previous validation of this effect at protein level (Poujade et al 2018), we are reassure of the impact of WSB-1 on the target gene transcripts is robust.

The use of *in vitro* models, such as established cancer cell lines, might justify some of the differences and unexpected results observed, in relation to literature research on patient bone metastasis breast cancer cells. For example, TNFRSF11B was of high relevance during the literature research but during the laboratory experiments there was no significance found when WSB1 was downregulated, table 14. This is due to experiments *in vitro* not replicating the complexity of a tumour (Clarke et al., 2021). Within a tumour that has metastasised to the bone, multiple factors within the bone matrix influence the progression of the tumour such as cytokines produced by the stromal and immune cells, such as insulin growth factor and platelet-derived growth factor (Azim et al., 2012). All these factors mediate cellular interactions such as proliferation, and angiogenesis and stimulate essential signalling pathways that would not happen *in vitro*. An example of how the microenvironment can affect how and what genes are elevated can be seen in work by Gan et al., (2016). Gan et al., (2016) evaluated and concluded that tumour-associated macrophages assist in metastasis through enhancing COX-2-mediated intercellular communication and also inducing MMP9 expression in breast cancer cells. Although seen as a study limitation, *in vitro* experimental models are however an acceptable first line of exploration within a new line of research (Clarke et al., 2021).

It is also possible that results were not expected in regard to the patient dataset when compared to the qPCR results (table 14) due to the patient dataset being analysed using patient samples containing a variety of cell types, not just tumour cells. Using only one breast cancer cell type, MDA-IV, which is a bone homing cline derived from the MDA-MB-231 TNBC cell line, limits the direct comparison of these two methods. As research progresses, it would be interesting to perform patient dataset analysis where patient samples are separated by

subtypes and analyse if the correlations observed with *WSB1* levels within subtypes would change from all patient cohorts.

When comparing findings from the RNA-sequencing data and the target gene expression validation through qPCR, it may be possible that we did not see an association within the results due to the cell type. The breast cancer cells used for the RNA sequence were the MDA-MB-231 TNBC breast cancer cell line, whereas for qPCR validation the MDA-IV bone homing clone of MDA-MB-231 was used (Nutter et al., 2014). It is important to use these cells as it has been identified that different gene and protein expression is apparent with bone-homing or colonisation cells at secondary tumour sites (Lawson et al., 2009; Ding et al., 2010). The bone is the most found secondary site of breast cancer, an ever more apparent reason as to why research in this area is crucial (Shah et al., 2023). The MDA-IV cell line was created by six cycles of IV intracardiac injections of the MDA-MB-231 cell line, where, once situated in the bloodstream, the arterial pressure traps the cells in the capillaries and transports them to the bone environment (Bellahcene et al., 2007).

MDA-MB-231 is a triple-negative cell line commonly used in bone metastasis research as they are prone to seed in the bone, developing osteolytic lesions as early as 3 weeks post-inoculation in studies involving mice (Wright et al,. 2016). MDA-MB-231 were not used in this research design due to lacking important molecules that are required in bone homing such as CXCR4 and the upregulation of IL1β, because of which the MDA-IV model was prefered (Parker and Sukumar, 2003; Nutter et al., 2014). Studies like this show that bone-homing cells alter specific genes in the early stages of breast cancer bone metastasis and that using these parental cells might not offer as informative results as would using a bone-homing cell (Wright et al,. 2016).

4.3 Future work

Results from this study have demonstrated that WSB1 could play an important role in breast cancer and bone metastasis. With this initial insight, there are many ways in which this research gap could be explored in future work, for example, flow adhesion assays could validate the metastatic potential and the recent development of organ-on-a-chip methods, allowing researchers to analyse cancer cells behaviour and signalling pathways while using

recreated microenvironment of the tissue being studied (Slay et al., 2021). Work validating a WSB1 inhibitor in breast cancer cells should be carried out due to previous success in human osteogenic sarcoma and non-small cell lung cancer cell lines (Che et al., 2021).

4.3.1 Analysis through Flow Adhesion Assays

Cell adhesion is essential for cell communication, regulation, behaviour, and metastatic potential (Khalili et al., 2015). Within this study, we have found key components that are affected by siWSB1 in regards to cell adhesion, such as the downregulation of *ICAM1.* A protocol to analyse the adhesion properties of MDA-231-IV cell line with siWSB1 could be utilised in future work. These include wash assays, centrifugation, flow chambers and spinning disks. Wash assay protocols involve static cultured cells and involve washing the cells one or more times, then analysing the adhered cells and non-adhered, this would indicate if gene expression is different and what that results in. Although a simple protocol, it can produce poor reproducibility, and insensitive data (Chen et al., 2010; Park et al., 2011). Spinning disks and flow chambers utilise shear stress to test the adhesion strength of the cells (Kang et al., 2016). These methods favour reproducibility, and adjustability in stress and demonstrate real-time cell detachment but can be labour-intensive and the flow of the chamber can fall to the centre of the dish, decreasing stress on the cells on the edge (Ungai-Salanki et al., 2019).

4.3.2 Metastasis-on-a-chip

Metastasis-on-a-chip is a new approach to understanding crosstalk between cancer cells and the specific niche of cancer metastasis. Organ-on-a-chip methods are three-dimensional, in vitro models with a continuous supply of cultured medium, they are used to recreate the microenvironment of the tissue being studied (Slay et al., 2021), for example, the microenvironment of the bone-on-a-chip can consist of mesenchymal stromal cells, osteoblasts, osteoclasts and hydroxyapatite (Slay et al., 2021). Conceição et al. (2021) and Zhang et al. (2022) state that on-the-chip technology is becoming a useful tool to mimic and control multiple factors that cannot be replicated in 2D in vitro methods. Adopting this technique for future work of siWSB1 could allow more realistic observations between invasion, intravasation, extravasation and angiogenesis within bone metastasis. Additionally to siRNA protocols, CRISPR technology of WSB-1 knockout could also be used *in vitro.* These

reports would show the effects WSB-1 has on bone metastasis transcriptional pathways, proliferation, and further metastasis.

4.3.3 Co-culture experiments with bone niche cells

Co-culture experiments are commonly used within cancer research, these methods are used to better understand the interactions between cancer cells and the host sites. Fitzgerald et al. (2016) have used this technique to better understand prostate cancer bone metastasis. Here they culture prostate cancer cells and osteoblast bone cells together and found that within doing this, cell proliferation was reduced by 50%. Bersini et al. (2014) used a breast cancer cell line (MDA-MB-231) tri-cultured with bone marrow-derived human mesenchymal stem cells and endothelial cells. Monitoring the breast cancer behaviour in regards to migration and proliferation. It was found that within the bone-mimicking microenvironment, the breast cancer cells migrated significantly further than the control. An attempt to expose HS5 bone mesenchymal cells to conditioned MDA-IV media on which WSB-1 was knocked down or not was performed. Due to time limitations, this experiment was only performed once and therefore can not be analysed statistically. This method would need improvements before being repeated due to several limitations. One limitation to address would be when imaging and counting the cells the whole culture vessel has to be taken out of the required incubators and hypoxia chamber and therefore be exposed to alternative temperatures and oxygen conditions. This may have affected the overall survival and proliferation of cells needed for counts in later days. A simple solution to this would have been to seed the cells into different culture wells on different trays, allowing for the removal of only the necessary cells that would have been imaged and counted on that day. Another limitation to consider to cell seeding and counting throughout this study would be the inaccuracy of manual counting using the haemocytometer. Manual cell counting is open to potential personal bias as counts would alter from user to user, there are also complications when loading the chambers as uneven distribution can also affect the overall counts and therefore seeding numbers would alter from repeat to repeat.

4.3.4 Invasion and migration assays

As siWSB1 had such an impact of *MMP9* gene expression, another useful step would be to compare the effects of WSB-1 modulation in the cell line MDA-IV using wound healing assays. Wound healing assays are commonly used to investigate cancer cell behaviour. An in vitro

technique used to evaluate cell migration and proliferation. The method includes scratching an area of the confluent monolayer of the observing cells and monitoring how the wound closes (Almeida et al., 2020). Performing cell invasion essays such as transwell assays could also monitor migration behaviours with the tumour cells, as these are used to monitor cell movement through extracellular matrices (Justus et al., 2014). This could be a useful method to use with the reduction of *ICAM1* expression with siSWB1. It could be of interest to see if the reduction in gene expression through siWSB1 corresponds to cell behaviour and migration in vitro in MDA-IV cells as previously observed for MDA-MB-231 cells (Poujade et al 2018).

4.3.5 Modelling the role of WSB-1 in the bone microenvironment

An additional consideration when planning future experiments would be to stimulate a more 'bone-like' environment. Shah et al. (2023) studied the effects of 2D cultured MDA-MB-231 cells and 3D cultured, looking specifically at the expression of *PTLHL*/PTHrP and IL-6. The authors found that the expression levels of both proteins when cells were cultured in 2D control conditions made no significant difference, however, when changing the environment, to stimulate the stiffness of the bone using hydrogel beads, the findings were of significance. A similar, more relevant technique was developed in a paper by Lefley et al. (2019) that represents bone metastatic breast cancer. The method favours using bone implants from human donors placed *in vivo* to carry out research of the modelling of breast cancer bone metastasis using clinically relevant mouse models. This experiment by Lefley et al. (2019) revealed that using mouse models, injected with MDA-MD-231 cells, specifically inhibiting IL-1β signalling, significantly reduced bone metastasis. Additionally, to utilising bone modelling microenvironments, CRISPR technology of WSB-1 knockout could be explored in these experiments to further indicate the signalling pathway that WSB-1 may regulate. Injecting genetically modified bone-homing breast cancer cells into sites required on mice, cells that have been modified to have deleted the *WSB1* gene. The tumours would then be harvested, and transcripts analysed, and tumour growth reported. These reports would show the effects WSB-1 has on bone metastasis transcriptional pathways, proliferation, and further metastasis *in vivo*.

4.4 Clinical relevance of WSB-1 in bone metastasis: target and biomarker

Studies from our lab indicate that WSB-1 plays an important role in breast cancer metastasis (Poujade et al 2018), and the present study indicates a potential further role in bone metastasis. Therefore, future work exploring the use of a WSB-1 inhibitor should be considered. There is potential that WSB-1 could be explored to be used as a prognostic biomarker, with high levels indicating to drive metastatic potential. Recent studies including this one open the discussion for WSB-1 to also be used as a predictive biomarker. With the potential to inhibit WSB-1 using IL1β antibodies, and gene inhibitors such as *ICAM1, MMP1, TGFBR1 and PTHLH*.

Che et al. (2021) used a phenotypic screening model to identify WSB-1 inhibitors, where they identified that compound 4 (WSB1 degrader 1) which showed responsibility for migration inhibitory activity against cells overexpressing WSB1. WSB1 degrader 1 has already proposed the potential of anti-migratory effects in KHOS and H460 cell lines (Che et al., 2021). In KHOS cells, WSB1 degrader 1 showed promising inhibition of cell migration under both hypoxic and normoxic conditions, additionally in hypoxia, elevated levels of RhoGDI2 protein were observed (Che et al., 2021). This in turn reverses the expression of downstream F-actin and the formation of membrane ruffles, reporting to enhance the disturbance of the migration capacity of the cancer cells. Results observed from wound-healing essays of H1299-WSB1 and A2780 (A2780-WT) cells were also seen to be significant to the inhibition of the WSB1 Degrader 1 (Che et al., 2021).

A former colleague (Olivia Dean, data not published) is currently validating this WSB-1 inhibitor in MDA-231-IV cells lines. In addition to the research by Che et al. (2021), Weng et al. (2022) also applied virtual screening and molecular dynamic solutions to their research and found that compound G490-0341 indicated a stable structure and therefore could also be a favourable compound for future WSB-1 inhibitor developments.

WSB-1 also has the potential to become a prognostic biomarker, with high levels indicating to drive metastatic potential and be a poor prognostic indicator for relapse-free survival in breast cancer patients (Kim et al., 2015; Poujade et al., 2018; Che et al., 2021). Further investigation of the correlation in protein expression in patient samples with breast cancer bone metastasis would further support this theory. IL1β has already been reported to be a

potential biomarker for breast cancer in the primary site, with high expression levels indicating a heightened risk for metastasis to the bone (Tulotta and Ottewell, 2018).

Findings within this study open an interesting discussion about whether high levels of WSB-1 could also indicate high IL1β expression in breast cancer patients and therefore highlight chances of primary tumour cells migrating to the bone. Therefore, future work discovering the role of WSB-1 could indicate the protein to be used as a predictive biomarker, indicating which treatment measures could be used on a patient. For example, patients with overexpression of WSB-1 could be treated with IL1β antibodies. IL1β antibody studies are currently recruiting patients although *in vivo* studies have shown that the inhibition of IL1β reduces primary tumour growth and metastasis (Tulotta et al., 2021).

In addition to IL1β antibodies, gene expression inhibitors such as *ICAM1, MMP1, TGFBR1 and PTHLH* could be utilised with patients expressing high levels of WSB-1. Particularly, targeting the expression of WSB-1 through a *PTHLH* inhibitor could disturb the RANKL pathway and therefore inhibit the vicious cycle (Li et al., 2022; Swami et al., 2023). In particular, Swami et al. (2023) found that using PTHrP inhibitors *in vivo* reduced skeletal tumour burden by 87% and could provide a therapeutic approach against TNBC bone metastasis. Likewise, Denosumab is a clinically successful human monoclonal antibody directed against RANKL (Kearns et al., 2008; Clézardin, 2011). WSB-1 is associated with increased RANKL pathways, this drug could hypothetically be used in patients expressing high levels of WSB-1.

4.5 Conclusions

To conclude, this study suggests that WSB-1 might play an important role in bone metastasis biology by enhancing the vicious cycle of bone metastasis, but further work is needed to validate this. Future work includes work on potential WSB-1 inhibitors and the use of WSB-1 as a clinical biomarker to improve patient outcomes, but extensive research will have to be carried out before coming to a definitive conclusion. There is also the potential to use clinically available inhibitors for such genes that have been highlighted as having a significant relation to WSB-1.

References

Abdulkhaleq, L.A., Assi, M.A., Abdullah, R., Zamri-Saad, M., Taufiq-Yap, Y.H. and Hezmee, M.N.M., (2018). The crucial roles of inflammatory mediators in inflammation: A review. *Veterinary world*, 11(5), p.627.

Afonina, I.S., Zhong, Z., Karin, M. and Beyaert, R., (2017). Limiting inflammation—the negative regulation of NF-κB and the NLRP3 inflammasome. *Nature immunology*, 18(8), pp.861-869.

Aggarwal, V., Tuli, H.S., Varol, A., Thakral, F., Yerer, M.B., Sak, K., Varol, M., Jain, A., Khan, M.A. and Sethi, G., (2019). Role of reactive oxygen species in cancer progression: molecular mechanisms and recent advancements. *Biomolecules*, 9(11), p.735.

Aguilar-Cazares, D., Chavez-Dominguez, R., Carlos-Reyes, A., Lopez-Camarillo, C., Hernadez de la Cruz, O.N. and Lopez-Gonzalez, J.S., (2019). Contribution of angiogenesis to inflammation and cancer. *Frontiers in oncology*, 9, p.1399.

Agyemang, K., Johansen, A.M., Barker, G.W., Pennison, M.J., Sheffield, K., Jimenez, H., Blackman, C., Sharma, S., Fordjour, P.A., Singh, R. and Cook, K.L., (2022). TGFBR1* 6A as a modifier of breast cancer risk and progression: advances and future prospects. *NPJ Breast Cancer*, *8*(1), p.84.

Ahmad Khalili, A. and Ahmad, M.R., (2015). A review of cell adhesion studies for biomedical and biological applications. *International journal of molecular sciences*, 16(8), pp.18149-18184.

Alemayehu, M., Dragan, M., Pape, C., Siddiqui, I., Sacks, D.B., Di Guglielmo, G.M., Babwah, A.V. and Bhattacharya, M., (2013). β-Arrestin2 regulates lysophosphatidic acid-induced human breast tumor cell migration and invasion via Rap1 and IQGAP1. *PloS one*, 8(2), p.e56174.

Ali, S., Rasool, M., Chaoudhry, H., Pushparaj, P.N., Jha, P., Hafiz, A., Mahfooz, M., Sami, G.A., Kamal, M.A., Bashir, S. and Ali, A., (2016). Molecular mechanisms and mode of tamoxifen resistance in breast cancer. *Bioinformation*, 12(3), p.135.

Allocca, G., Hughes, R., Wang, N., Brown, H.K., Ottewell, P.D., Brown, N.J. and Holen, I., (2019). The bone metastasis niche in breast cancer: potential overlap with the haematopoietic stem cell niche in vivo. *Journal of bone oncology*, 17, p.100244.

Almeida, V.M., Bezerra, M.A., Nascimento, J.C. and Amorim, L.M.F., (2020). Anticancer drug screening: Standardization of in vitro wound healing assay. *Jornal Brasileiro de Patologia e Medicina Laboratorial*, 55, pp.606-619.

Ara, T. and DeClerck, Y.A., (2010). Interleukin-6 in bone metastasis and cancer progression. *European journal of cancer*, 46(7), pp.1223-1231.

Archange, C., Nowak, J., Garcia, S., Moutardier, V., Calvo, E.L., Dagorn, J.C. and Iovanna, J.L., (2008). The WSB1 gene is involved in pancreatic cancer progression. *PloS one*, 3(6), p.e2475.

Arciero, C.A., Guo, Y., Jiang, R., Behera, M., O'Regan, R., Peng, L. and Li, X., (2019). ER+/HER2+ breast cancer has different metastatic patterns and better survival than ER−/HER2+ breast cancer. *Clinical breast cancer*, 19(4), pp.236-245.

Augustine, H., Munro, M., Adatia, F., Webster, M. and Fielden, M., (2014). Treatment of ocular metastasis with anti-VEGF: a literature review and case report. *Canadian Journal of Ophthalmology*, 49(5), pp.458-463.

Azim, H.A., Kamal, N.S. and Azim Jr, H.A., (2012). Bone metastasis in breast cancer: the story of RANK-ligand. *Journal of the Egyptian National Cancer Institute*, *24*(3), pp.107-114.

Badwe, R., Hawaldar, R., Nair, N., Kaushik, R., Parmar, V., Siddique, S., Budrukkar, A., Mittra, I. and Gupta, S., (2015). Locoregional treatment versus no treatment of the primary tumour in metastatic breast cancer: an open-label randomised controlled trial. *The lancet oncology*, *16*(13), 1380-1388.

Bahhnassy, A., Mohanad, M., Shaarawy, S., Ismail, M.F., El Bastawisy, A., Ashmawy, A.M. and Zekri, A.R., (2015). Transforming growth factor β, insulin like growth factor I/insulin like growth factor I receptor and vascular endothelial growth factor A: Prognostic and predictive markers in triple negative and non triple negative breast cancer. *Molecular Medicine Reports*, 12(1), pp.851-864.

Bahmaee, H., Owen, R., Boyle, L., Perrault, C.M., Garcia-Granada, A.A., Reilly, G.C. and Claeyssens, F., (2020). Design and evaluation of an osteogenesis-on-a-chip microfluidic device incorporating 3D cell culture. *Frontiers in bioengineering and biotechnology, 8, p.557111.*

Balma, M., Liberini, V., Racca, M., Laudicella, R., Bauckneht, M., Buschiazzo, A., Nicolotti, D.G., Peano, S., Bianchi, A., Albano, G. and Quartuccio, N., (2022). Non-conventional and investigational PET radiotracers for breast cancer: a systematic review. *Frontiers in Medicine*, 9, p.881551.

Bellahcene, A., Bachelier, R., Detry, C., Lidereau, R., Clezardin, P. and Castronovo, V., (2007). Transcriptome analysis reveals an osteoblast-like phenotype for human osteotropic breast cancer cells. *Breast cancer research and treatment*, *101*, pp.135-148.

Bendre, M.S., Gaddy-Kurten, D., Mon-Foote, T., Akel, N.S., Skinner, R.A., Nicholas, R.W. and Suva, L.J., (2002). Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis in vivo. *Cancer research*, 62(19), pp.5571-5579.

Benita, Y., Kikuchi, H., Smith, A.D., Zhang, M.Q., Chung, D.C., and Xavier, R.J. (2009). An integrative genomics approach identifies Hypoxia Inducible Factor-1 (HIF-1)-target genes that form the core response to hypoxia. *Nucleic Acids Res*. 37, 4587–4602.

Bersini, S., Jeon, J.S., Dubini, G., Arrigoni, C., Chung, S., Charest, J.L., Moretti, M. and Kamm, R.D., (2014). A microfluidic 3D in vitro model for specificity of breast cancer metastasis to bone. *Biomaterials*, *35*(8), pp.2454-2461.

Bhandari V, Hoey C, Liu LY, Lalonde E, Ray J, Livingstone J, Lesurf R, Shiah YJ, Vujcic T, Huang X, Espiritu SMG, Heisler LE, Yousif F, Huang V, Yamaguchi TN, Yao CQ, Sabelnykova VY, Fraser M, Chua MLK, van der Kwast T, Liu SK, Boutros PC, Bristow RG. Borsig, L., Läubli, H., Boffetta, P. and Hainaut, P., (2019). Cell adhesion during tumorigenesis and metastasis.

Boldrini, L. and Bardi, M., (2023). WSB1 Involvement in Prostate Cancer Progression. Genes, 14(*8*), p.1558.

Breast Cancer. (2021). Some Triple-Negative Breast Cancers Are More Aggressive Than Others. [online] Available at: <https://www.breastcancer.org/research-news/some-triple-negative-breast-cancers-more-aggressive> [Accessed 16 May 2022].

Brunt, A.M., Haviland, J.S., Wheatley, D.A., Sydenham, M.A., Alhasso, A., Bloomfield, D.J., Chan, C., Churn, M., Cleator, S., Coles, C.E. and Goodman, A., (2020). Hypofractionated breast radiotherapy for 1 week versus 3 weeks (FAST-Forward): 5-year efficacy and late normal tissue effects results from a multicentre, non-inferiority, randomised, phase 3 trial. *The Lancet*, 395(10237), pp.1613-1626.

Burslem, G.M., Kyle, H.F., Nelson, A., Edwards, T.A. and Wilson, A.J., (2017). Hypoxia inducible factor (HIF) as a model for studying inhibition of protein–protein interactions. *Chemical science*, 8(6), pp.4188-4202.

Busk, M., Overgaard, J. and Horsman, M.R., (2020), November. Imaging of tumor hypoxia for radiotherapy: current status and future directions. *In Seminars in nuclear medicine* (Vol. 50, No. 6, pp. 562-583). WB Saunders.

Cancer Research UK, 2022 https://www.cancerresearchuk.org/health-professional/cancer-statistics/mortality/common-cancers-compared#heading-Two, Accessed July 2023.

Cancer Research UK, https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer#heading-Zero, Accessed [September] [2021].

Cancer Research uk. (2020). Survival for breast cancer | Breast Cancer | Cancer Research UK. [online] Available at: <https://www.cancerresearchuk.org/about-cancer/breast-cancer/survival> [Accessed 16 May 2022].

Cao, J., Wang, Y., Dong, R., Lin, G., Zhang, N., Wang, J., Lin, N., Gu, Y., Ding, L., Ying, M. and He, Q., (2015). Hypoxia-induced WSB1 promotes the metastatic potential of osteosarcoma cells. *Cancer Research*, *75*(22), 4839-4851.

Cao, J., Wang, Y., Dong, R., Lin, G., Zhang, N., Wang, J., Lin, N., Gu, Y., Ding, L., Ying, M. and He, Q., (2015). Hypoxia-induced WSB1 promotes the metastatic potential of osteosarcoma cells. *Cancer Research*, 75(22), pp.4839-4851.

Cao, Y. and Zhang, J., (2019). Protein Assay Based on Protein–Small Molecule Interaction. *In Nano-Inspired Biosensors for Protein Assay with Clinical Applications* (pp. 187-205). Elsevier.

Casimiro, S., Luis, I., Fernandes, A., Pires, R., Pinto, A., Gouveia, A.G., Francisco, A.F., Portela, J., Correia, L. and Costa, L., (2012). Analysis of a bone metastasis gene expression signature in patients with bone metastasis from solid tumors. *Clinical & experimental metastasis*, 29, pp.155-164.

Certo, M., Tsai, C.H., Pucino, V., Ho, P.C. and Mauro, C., (2021). Lactate modulation of immune responses in inflammatory versus tumour microenvironments. *Nature Reviews Immunology*, 21(3), pp.151-161.

Chappell, J.C., Payne, L.B. and Rathmell, W.K., (2019). Hypoxia, angiogenesis, and metabolism in the hereditary kidney cancers. *The Journal of clinical investigation*, 129(2), pp.442-451.

Chaturvedi, P., Gilkes, D.M., Takano, N. and Semenza, G.L., (2014). Hypoxia-inducible factor-dependent signaling between triple-negative breast cancer cells and mesenchymal stem cells promotes macrophage recruitment. *Proceedings of the National Academy of Sciences*, 111(20), pp.E2120-E2129.

Che, J., Jin, Z., Yan, F., You, J., Xie, J., Chen, B., Cheng, G., Zhu, H., He, Q., Hu, Y. and Yang, B., (2021). Discovery of 5, 6-Bis (4-methoxy-3-methylphenyl) pyridin-2-amine as a WSB1 degrader to inhibit cancer cell metastasis. *Journal of Medicinal Chemistry*, 64(12), pp.8621-8643.

Chen, M., Wu, C., Fu, Z. and Liu, S., (2022). ICAM1 promotes bone metastasis via integrin-mediated TGF-β/EMT signaling in triple-negative breast cancer. *Cancer Science*, 113(11), p.3751.

Chen, W., Hoffmann, A.D., Liu, H. and Liu, X., (2018). Organotropism: new insights into molecular mechanisms of breast cancer metastasis. *NPJ precision oncology*, 2(1), p.4.

Chen, X., Luther, G., Zhang, W., Nan, G., Wagner, E.R., Liao, Z., Wu, N., Zhang, H., Wang, N., Wen, S. and He, Y., (2013). The EF hand calcium-binding protein S100A4 regulates the proliferation, survival and differentiation potential of human osteosarcoma cells. *Cellular Physiology and Biochemistry*, 32(4), pp.1083-1096.

Chen, X., Wang, M., Yang, X., Wang, Y., Yu, L., Sun, J. and Ding, J., (2019). Injectable hydrogels for the sustained delivery of a HER2-targeted antibody for preventing local relapse of HER2+ breast cancer after breast-conserving surgery. *Theranostics*, 9(21), p.6080.

Chen, X., Zhao, F., Zhang, H., Zhu, Y., Wu, K. and Tan, G., (2015). Significance of TLR4/MyD88 expression in breast cancer. *International journal of clinical and experimental pathology*, 8(6), p.7034.

Chiorcea-Paquim, A.M. and Oliveira-Brett, A.M., (2023). Electrochemistry of chemotherapeutic alkylating agents and their interaction with DNA. *Journal of pharmaceutical and biomedical analysis*, 222, p.115036.

Christopoulos, P.F., Msaouel, P. and Koutsilieris, M., (2015). The role of the insulin-like growth factor-1 system in breast cancer. *Molecular cancer*, 14(1), pp.1-14.

Clarke, R., Jones, B.C., Sevigny, C.M., Hilakivi-Clarke, L.A. and Sengupta, S., (2021). Experimental models of endocrine responsive breast cancer: Strengths, limitations, and use. *Cancer Drug Resistance*, *4*(4), p.762.

Clézardin, P., (2011). Therapeutic targets for bone metastases in breast cancer. *Breast Cancer Research,* 13, pp.1-9.

Clézardin, P., Coleman, R., Puppo, M., Ottewell, P., Bonnelye, E., Paycha, F., Confavreux, C.B. and Holen, I., (2021). Bone metastasis: Mechanisms, therapies, and biomarkers. *Physiological reviews*, *101*(3), pp.797-855.

Coleman, R., (2020). Bisphosphonates and breast cancer–from cautious palliation to saving lives. *Bone*, 140, p.115570.

Coleman, R.E., Brown, J. and Holen, I., (2020). Bone metastases. *Abeloff's Clinical Oncology*, 809-830.

Conceição, F., Sousa, D.M., Loessberg-Zahl, J., Vollertsen, A.R., Neto, E., Søe, K., Paredes, J., Leferink, A. and Lamghari, M., (2022). A metastasis-on-a-chip approach to explore the sympathetic modulation of breast cancer bone metastasis. *Materials Today Bio*, 13, p.100219.

Conte, A., Valente, V., Paladino, S. and Pierantoni, G.M., (2023). HIPK2 in cancer biology and therapy: Recent findings and future perspectives. Cellular Signalling, 101, p.110491.

Cortes, J., Rugo, H.S., Cescon, D.W., Im, S.A., Yusof, M.M., Gallardo, C., Lipatov, O., Barrios, C.H., Perez-Garcia, J., Iwata, H. and Masuda, N., (2022). Pembrolizumab plus chemotherapy

in advanced triple-negative breast cancer. *New England Journal of Medicine*, 387(3), pp.217-226.

Curtis, C., Shah, S.P., Chin, S.F., Turashvili, G., Rueda, O.M., Dunning, M.J., Speed, D., Lynch, A.G., Samarajiwa, S., Yuan, Y. and Gräf, S., (2012). The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*, 486(7403), pp.346-352.

Darb-Esfahani, S., Denkert, C., Stenzinger, A., Salat, C., Sinn, B., Schem, C., Endris, V., Klare, P., Schmitt, W., Blohmer, J.U. and Weichert, W., (2016). Role of TP53 mutations in triple negative and HER2-positive breast cancer treated with neoadjuvant anthracycline/taxane-based chemotherapy. *Oncotarget*, 7(42), p.67686.

de la Cruz-López, K.G., Castro-Muñoz, L.J., Reyes-Hernández, D.O., García-Carrancá, A. and Manzo-Merino, J., (2019). Lactate in the regulation of tumor microenvironment and therapeutic approaches. *Frontiers in oncology*, 9, p.1143.

Dhillon, A.S., Hagan, S., Rath, O. and Kolch, W., (2007). MAP kinase signalling pathways in cancer. *Oncogene*, 26(22), pp.3279-3290.

Dinarello, C.A., (2015). The history of fever, leukocytic pyrogen and interleukin-1. *Temperature*, 2(1), pp.8-16.

Ding, L.I., Ellis, M.J., Li, S., Larson, D.E., Chen, K., Wallis, J.W., Harris, C.C., McLellan, M.D., Fulton, R.S., Fulton, L.L. and Abbott, R.M., (2010). Genome remodelling in a basal-like breast cancer metastasis and xenograft. *Nature*, 464(7291), pp.999-1005.

Eck, S.M., Hoopes, P.J., Petrella, B.L., Coon, C.I. and Brinckerhoff, C.E., (2009). Matrix metalloproteinase-1 promotes breast cancer angiogenesis and osteolysis in a novel in vivo model. *Breast cancer research and treatment*, 116, pp.79-90.

Emmanouilidi, A., Casari, I., Gokcen Akkaya, B., Maffucci, T., Furic, L., Guffanti, F., Broggini, M., Chen, X., Maxuitenko, Y.Y., Keeton, A.B. and Piazza, G.A., (2020). Inhibition of the lysophosphatidylinositol transporter ABCC1 reduces prostate cancer cell growth and sensitizes to chemotherapy. *Cancers*, 12(8), p.2022.

Erber, R. and Hartmann, A., (2020). Histology of luminal breast cancer. *Breast Care*, 15(4), pp.327-336.

Fernald, K. and Kurokawa, M., (2013). Evading apoptosis in cancer. *Trends in cell biology*, 23(12), pp.620-633.

Fico, F., Bousquenaud, M., Rüegg, C. and Santamaria-Martínez, A., (2019). Breast cancer stem cells with tumor-versus metastasis-initiating capacities are modulated by TGFBR1 inhibition. *Stem Cell Reports*, *13*(1), pp.1-9.

Filippi, M.D., (2016). Mechanism of diapedesis: importance of the transcellular route. *Advances in immunology*, 129, pp.25-53.

Forbes, C., Fayter, D., de Kock, S. and Quek, R.G., (2019). A systematic review of international guidelines and recommendations for the genetic screening, diagnosis, genetic counseling, and treatment of BRCA-mutated breast cancer. *Cancer management and research*, pp.2321-2337.

Gaete, D., Rodriguez, D., Watts, D., Sormendi, S., Chavakis, T. and Wielockx, B., (2021). HIF-Prolyl Hydroxylase Domain Proteins (PHDs) in Cancer—Potential Targets for Anti-Tumor Therapy?. *Cancers*, 13(5), p.988.

Gan, L., Qiu, Z., Huang, J., Li, Y., Huang, H., Xiang, T., Wan, J., Hui, T., Lin, Y., Li, H. and Ren, G., (2016). Cyclooxygenase-2 in tumor-associated macrophages promotes metastatic potential of breast cancer cells through Akt pathway. *International journal of biological sciences*, *12*(12), p.1533.

Gao, X., You, J., Gong, Y., Yuan, M., Zhu, H., Fang, L., Zhu, H., Ying, M., He, Q., Yang, B. and Cao, J., (2022). WSB1 regulates c-Myc expression through β-catenin signaling and forms a feedforward circuit. Acta Pharmaceutica Sinica B, 12(*3*), pp.1225-1239.

Gao, F.Y., Li, X.T., Xu, K., Wang, R.T. and Guan, X.X., (2023). c-MYC mediates the crosstalk between breast cancer cells and tumor microenvironment. Cell Communication and Signaling, 21(*1*), p.28.

Gao, J.J. and Swain, S.M., (2018). Luminal a breast cancer and molecular assays: a review. *The oncologist*, 23(5), pp.556-565.

Gaynor, N., Crown, J. and Collins, D.M., (2022), February. Immune checkpoint inhibitors: Key trials and an emerging role in breast cancer. In Seminars in cancer biology (Vol. 79, pp. 44-57). Academic Press.

Geeraerts, X., Fernandez-Garcia, J., Hartmann, F.J., de Goede, K.E., Martens, L., Elkrim, Y., Debraekeleer, A., Stijlemans, B., Vandekeere, A., Rinaldi, G. and De Rycke, R., (2021). Macrophages are metabolically heterogeneous within the tumor microenvironment. *Cell reports*, 37(13).

Garufi, A., D'Orazi, V., Pistritto, G., Cirone, M. and D'Orazi, G., (2023). The sweet side of HIPK2. Cancers, 15(*10*), p.2678.

Gilkes, D.M., Semenza, G.L. and Wirtz, D., (2014). Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nature Reviews Cancer*, 14(6), pp.430-439.

Goldberg, A.L., (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature*, 426(6968), pp.895-899.

Goutsouliak, K., Veeraraghavan, J., Sethunath, V., De Angelis, C., Osborne, C.K., Rimawi, M.F. and Schiff, R., (2020). Towards personalized treatment for early stage HER2-positive breast cancer. *Nature Reviews Clinical Oncology*, 17(4), pp.233-250.

Graham, K. and Unger, E., (2018). Overcoming tumor hypoxia as a barrier to radiotherapy, chemotherapy and immunotherapy in cancer treatment. *International journal of nanomedicine*, pp.6049-6058.

Gray, L.H., Conger, A., Ebert, M., Hornsey, S. and Scott, O.C.A., (1953). The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *The British journal of radiology*, 26(312), pp.638-648.

Guestini, F., Ono, K., Miyashita, M., Ishida, T., Ohuchi, N., Nakagawa, S., Hirakawa, H., Tamaki, K., Ohi, Y., Rai, Y. and Sagara, Y., (2019). Impact of Topoisomerase IIα, PTEN, ABCC1/MRP1, and KI67 on triple-negative breast cancer patients treated with neoadjuvant chemotherapy. *Breast cancer research and treatment*, 173, pp.275-288.

Hachim, M.Y., Hachim, I.Y., Dai, M., Ali, S. and Lebrun, J.J., (2018). Differential expression of TGFβ isoforms in breast cancer highlights different roles during breast cancer progression. *Tumor Biology*, 40(1), p.1010428317748254.

Haider, M.T., Ridlmaier, N., Smit, D.J. and Taipaleenmäki, H., (2021). Interleukins as mediators of the tumor cell—bone cell crosstalk during the initiation of breast cancer bone metastasis. *International Journal of Molecular Sciences*, 22(6), p.2898.

Hailemichael, Y., Johnson, D.H., Abdel-Wahab, N., Foo, W.C., Bentebibel, S.E., Daher, M., Haymaker, C., Wani, K., Saberian, C., Ogata, D. and Kim, S.T., (2022). Interleukin-6 blockade abrogates immunotherapy toxicity and promotes tumor immunity. *Cancer Cell*, *40*(5), pp.509-523.

Hanahan, D. and Weinberg, R.A., (2011). Hallmarks of cancer: the next generation. *cell*, 144(5), pp.646-674.

Hao, Z., Ma, Y., Wu, J., Li, X., Chen, H., Shen, J. and Wang, H., (2017). Osteocytes regulate osteoblast differentiation and osteoclast activity through Interleukin-6 under mechanical loading. *RSC advances*, 7(79), pp.50200-50209.

Heeke, A.L. and Tan, A.R., (2021). Checkpoint inhibitor therapy for metastatic triple-negative breast cancer. *Cancer and Metastasis Reviews*, 40(2), pp.537-547.

Hilton, D.J., Richardson, R.T., Alexander, W.S., Viney, E.M., Willson, T.A., Sprigg, N.S., Starr, R., Nicholson, S.E., Metcalf, D. and Nicola, N.A., (1998). Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proceedings of the National Academy of Sciences*, 95(1), pp.114-119.

Hiraga, T., Myoui, A., Hashimoto, N., Sasaki, A., Hata, K., Morita, Y., Yoshikawa, H., Rosen, C.J., Mundy, G.R. and Yoneda, T., (2012). Bone-derived IGF mediates crosstalk between bone and breast cancer cells in bony metastases. *Cancer research*, 72(16), pp.4238-4249.

Hirota, K., (2020). Basic biology of hypoxic responses mediated by the transcription factor HIFs and its implication for medicine. Biomedicines, 8(2), p.32.Hultsch, S., Kankainen, M., Paavolainen, L., Kovanen, R.M., Ikonen, E., Kangaspeska, S., Pietiäinen, V. and Kallioniemi, O., 2018. Association of tamoxifen resistance and lipid reprogramming in breast cancer. *BMC cancer*, 18, pp.1-14.

Hoadley KA, Yau C, Hinoue T, Wolf DM, Lazar AJ, Drill E, Shen R, Taylor AM, Cherniack AD, Thorsson V, Akbani R, Bowlby R, Wong CK, Wiznerowicz M, Sanchez-Vega F, Robertson AG, Schneider BG, Lawrence MS, Noushmehr H, Malta TM; Cancer Genome Atlas Network, Stuart JM, Benz CC, Laird PW.

Hompland, T., Fjeldbo, C.S. and Lyng, H., (2021). Tumor hypoxia as a barrier in cancer therapy: why levels matter. *Cancers*, 13(3), p.499.

Incorvati, J.A., Shah, S., Mu, Y. and Lu, J., (2013). Targeted therapy for HER2 positive breast cancer. *Journal of hematology & oncology*, 6(1), pp.1-9.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin Jr, W.G., (2001). HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*, 292(5516), pp.464-468.

Jeong, H., Kim, S., Hong, B.J., Lee, C.J., Kim, Y.E., Bok, S., Oh, J.M., Gwak, S.H., Yoo, M.Y., Lee, M.S. and Chung, S.J., (2019). Tumor-associated macrophages enhance tumor hypoxia and aerobic glycolysis. *Cancer research*, 79(4), pp.795-806.

Jing, X., Yang, F., Shao, C., Wei, K., Xie, M., Shen, H. and Shu, Y., (2019). Role of hypoxia in cancer therapy by regulating the tumor microenvironment. *Molecular cancer*, 18, pp.1-15.

Johnstone, C.N., Chand, A., Putoczki, T.L. and Ernst, M., (2015). Emerging roles for IL-11 signaling in cancer development and progression: Focus on breast cancer. *Cytokine & growth factor reviews,* 26(5), pp.489-498.

Justus, C.R., Leffler, N., Ruiz-Echevarria, M. and Yang, L.V., (2014). In vitro cell migration and invasion assays. *JoVE (Journal of Visualized Experiments*), (88), p.e51046.

Kang, J., Choi, Y.J., Seo, B.Y., Jo, U., Park, S.I., Kim, Y.H. and Park, K.H., (2019). A Selective FGFR inhibitor AZD4547 suppresses RANKL/M-CSF/OPG-dependent ostoclastogenesis and breast cancer growth in the metastatic bone microenvironment. *Scientific reports*, 9(1), p.8726.

Kang, S.A., Bajana, S. and Tanaka, T., (2016). In vitro flow adhesion assay for analyzing shear-resistant adhesion of metastatic cancer cells to endothelial cells. *Bio-protocol*, 6(4), pp.e1731-e1731.

Karavitis, J., Hix, L.M., Shi, Y.H., Schultz, R.F., Khazaie, K. and Zhang, M., (2012). Regulation of COX2 expression in mouse mammary tumor cells controls bone metastasis and PGE2-induction of regulatory T cell migration.

Kearns, A.E., Khosla, S. and Kostenuik, P.J., (2008). Receptor activator of nuclear factor κB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocrine reviews,* 29(2), pp.155-192.

Kelly, B., Tannahill, G.M., Murphy, M.P. and O'Neill, L.A., (2015). Metformin inhibits the production of reactive oxygen species from NADH: ubiquinone oxidoreductase to limit induction of interleukin-1β (IL-1β) and boosts interleukin-10 (IL-10) in lipopolysaccharide (LPS)-activated macrophages. *Journal of biological chemistry*, *290*(33), pp.20348-20359.

Khorshidi, S., Younesi, S. and Karkhaneh, A., (2022). Peroxide mediated oxygen delivery in cancer therapy. *Colloids and Surfaces B: Biointerfaces*, p.112832.

Kiechl, S., Schramek, D., Widschwendter, M., Fourkala, E.O., Zaikin, A., Jones, A., Jaeger, B., Rack, B., Janni, W., Scholz, C. and Willeit, J., (2017). Aberrant regulation of RANKL/OPG in women at high risk of developing breast cancer. *Oncotarget*, *8*(3), p.3811.

Kim, H.N., Xiong, J., MacLeod, R.S., Iyer, S., Fujiwara, Y., Cawley, K.M., Han, L., He, Y., Thostenson, J.D., Ferreira, E. and Jilka, R.L., (2020). Osteocyte RANKL is required for cortical bone loss with age and is induced by senescence. *JCI insight*, 5(19).

Kim, J.J., Lee, S.B., Jang, J., Yi, S.Y., Kim, S.H., Han, S.A., Lee, J.M., Tong, S.Y., Vincelette, N.D., Gao, B. and Yin, P., (2015). WSB1 promotes tumor metastasis by inducing pVHL degradation. *Genes & development*, *29*(21), 2244-2257.

Kim, S., Lee, Y. and Koo, J.S., (2015). Differential expression of lipid metabolism-related proteins in different breast cancer subtypes. *PloS one*, 10(3), p.e0119473.

Knüpfer, H. and Preiß, R., (2007). Significance of interleukin-6 (IL-6) in breast cancer. *Breast cancer research and treatment,* 102, pp.129-135.

Koblinski, J.E., Kaplan-Singer, B.R., VanOsdol, S.J., Wu, M., Engbring, J.A., Wang, S., Goldsmith, C.M., Piper, J.T., Vostal, J.G., Harms, J.F. and Welch, D.R., (2005). Endogenous

osteonectin/SPARC/BM-40 expression inhibits MDA-MB-231 breast cancer cell metastasis. *Cancer research*, 65(16), pp.7370-7377.

Kodama, J. and Kaito, T., (2020). Osteoclast multinucleation: review of current literature. *International journal of molecular sciences*, 21(16), p.5685.

Koh, M.Y., Lemos Jr, R., Liu, X. and Powis, G., (2011). The hypoxia-associated factor switches cells from HIF-1α-to HIF-2α-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. *Cancer research*, 71(11), pp.4015-4027.

Koh, Y.Q., Mitchell, M.D., Almughlliq, F.B., Vaswani, K. and Peiris, H.N., (2018). Regulation of inflammatory mediator expression in bovine endometrial cells: effects of lipopolysaccharide, interleukin 1 beta, and tumor necrosis factor alpha. *Physiological reports*, *6*(9), p.e13676.

Korpal, M. and Kang, Y., (2010). Targeting the transforming growth factor-β signalling pathway in metastatic cancer. *European journal of cancer*, 46(7), pp.1232-1240.

Koujan, S.E., Gargarib, B.P. and Khalili, M., (2015). Matrix metalloproteinases and breast cancer. *Thrita*, 4(1).

Kumar, H. and Choi, D.K., (2015). Hypoxia inducible factor pathway and physiological adaptation: a cell survival pathway?. *Mediators of inflammation*, 2015.

Kung-Chun Chiu, D., Pui-Wah Tse, A., Law, C.T., Ming-Jing Xu, I., Lee, D., Chen, M., Kit-Ho Lai, R., Wai-Hin Yuen, V., Wing-Sum Cheu, J., Wai-Hung Ho, D. and Wong, C.M., (2019). Hypoxia regulates the mitochondrial activity of hepatocellular carcinoma cells through HIF/HEY1/PINK1 pathway. *Cell death & disease*, 10(12), p.934.

Kwon, M.J., (2023). Matrix metalloproteinases as therapeutic targets in breast cancer. *Frontiers in Oncology*, 12, p.1108695.

Lawson, J.C., Blatch, G.L. and Edkins, A.L., (2009). Cancer stem cells in breast cancer and metastasis. *Breast cancer research and treatment*, 118, pp.241-254.

Lee, J.H., Kim, B., Jin, W.J., Kim, J.W., Kim, H.H., Ha, H. and Lee, Z.H., (2014). Trolox inhibits osteolytic bone metastasis of breast cancer through both PGE2-dependent and independent mechanisms. *Biochemical pharmacology*, 91(1), pp.51-60.

Lee, J.J., Natsuizaka, M., Ohashi, S., Wong, G.S., Takaoka, M., Michaylira, C.Z., Budo, D., Tobias, J.W., Kanai, M., Shirakawa, Y. and Naomoto, Y., (2010). Hypoxia activates the cyclooxygenase-2–prostaglandin E synthase axis. *Carcinogenesis*, *31*(3), pp.427-434.

Lee, W.H., Choong, L.Y., Jin, T.H., Mon, N.N., Chong, S., Liew, C.S., Putti, T., Lu, S.Y., Harteneck, C. and Lim, Y.P., (2017). TRPV4 plays a role in breast cancer cell migration via Ca2+-dependent activation of AKT and downregulation of E-cadherin cell cortex protein. *Oncogenesis*, 6(5), pp.e338-e338.

Lee, S.H., Li, Y., Kim, H., Eum, S., Park, K. and Lee, C.H., (2022). The role of EZH1 and EZH2 in development and cancer. BMB reports, 55(*12*), p.595.

Li, J., Camirand, A., Zakikhani, M., Sellin, K., Guo, Y., Luan, X., Mihalcioiu, C. and Kremer, R., (2022). Parathyroid Hormone-Related Protein Inhibition Blocks Triple-Negative Breast Cancer Expansion in Bone Through Epithelial to Mesenchymal Transition Reversal. *JBMR plus*, 6(6), p.e10587.c

Li, P., Wu, M., Wang, J., Sui, Y., Liu, S. and Shi, D., (2016). NAC selectively inhibit cancer telomerase activity: A higher redox homeostasis threshold exists in cancer cells. *Redox biology*, 8, pp.91-97.

Li, Z., Schem, C., Shi, Y.H., Medina, D. and Zhang, M., (2008). Increased COX2 expression enhances tumor-induced osteoclastic lesions in breast cancer bone metastasis. *Clinical & experimental metastasis*, 25, pp.389-400.

Li, Z.H., Hu, P.H., Tu, J.H. and Yu, N.S., (2016). Luminal B breast cancer: patterns of recurrence and clinical outcome. *Oncotarget*, 7(40), p.65024.

Liang, Q., Ma, D., Gao, R.F. and Yu, K.D., (2020). Effect of Ki-67 expression levels and histological grade on breast cancer early relapse in patients with different immunohistochemical-based subtypes. *Scientific reports*, 10(1), p.7648.

Lin, T.C., (2019). The role of visfatin in cancer proliferation, angiogenesis, metastasis, drug resistance and clinical prognosis. *Cancer management and research*, pp.3481-3491.

Liu, B., Cui, J., Sun, J., Li, J., Han, X., Guo, J., Yi, M., Amizuka, N., Xu, X. and Li, M., (2016). Immunolocalization of MMP9 and MMP2 in osteolytic metastasis originating from MDA-MB-231 human breast cancer cells. *Molecular medicine reports*, 14(2), pp.1099-1106.

Liu, X., Fang, Y., Li, Y., Li, Y., Qi, L. and Wang, X., (2022). Pertuzumab combined with trastuzumab compared to trastuzumab in the treatment of HER2-positive breast cancer: A systematic review and meta-analysis of randomized controlled trials. *Frontiers in Oncology*, 12, p.894861.

Liu, X., Zhang, X., Peng, Z., Li, C., Wang, Z., Wang, C., Deng, Z., Wu, B., Cui, Y., Wang, Z. and Cui, C.P., (2020). Deubiquitylase OTUD6B governs pVHL stability in an enzyme-independent manner and suppresses hepatocellular carcinoma metastasis. *Advanced Science*, 7(8), p.1902040.

Loganadane, L.D., Berge, N., Legrand, C. and Fauvel-Lafeve, F., (1997). Endothelial cell proliferation regulated by cytokines modulates trombospondin-1 secretion into the subendothelium. Cytokine, 9(10), pp.740-746.

Loibl, S. and Gianni, L., 2017. HER2-positive breast cancer. *The Lancet*, 389(10087), pp.2415-2429.

Lombardi, A., Lazzeroni, R., Bersigotti, L., Vitale, V. and Amanti, C., (2021). The proper Ki-67 cut-off in hormone responsive breast cancer: a monoinstitutional analysis with long-term follow-up. *Breast Cancer: Targets and Therapy*, pp.213-217.

Lu, X., Wang, Q., Hu, G., Van Poznak, C., Fleisher, M., Reiss, M., Massagué, J. and Kang, Y., (2009). ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis. *Genes & development*, 23(16), pp.1882-1894.

Lundgren, K., Holm, C. and Landberg, G., (2007). Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer: Hypoxia and breast cancer: prognostic and therapeutic implications. *Cellular and Molecular Life Sciences*, 64, pp.3233-3247.

Manna, S. and Holz, M.K., (2016). Tamoxifen action in ER-negative breast cancer. *Signal transduction insights*, 5, pp.STI-S29901.

Marahleh, A., Kitaura, H., Ohori, F., Kishikawa, A., Ogawa, S., Shen, W.R., Qi, J., Noguchi, T., Nara, Y. and Mizoguchi, I., (2019). TNF-α directly enhances osteocyte RANKL expression and promotes osteoclast formation. *Frontiers in immunology*, 10, p.2925.

Marei, H.E., Althani, A., Afifi, N., Hasan, A., Caceci, T., Pozzoli, G., Morrione, A., Giordano, A. and Cenciarelli, C., (2021). p53 signaling in cancer progression and therapy. *Cancer cell international,* 21(1), pp.1-15.

Maroni, P., Bendinelli, P., Matteucci, E. and Desiderio, M.A., (2018). The therapeutic effect of miR-125b is enhanced by the prostaglandin endoperoxide synthase 2/cyclooxygenase 2 blockade and hampers ETS1 in the context of the microenvironment of bone metastasis. *Cell death & disease*, *9*(5), p.472.

Martin, T.J. and Johnson, R.W., (2021). Multiple actions of parathyroid hormone-related protein in breast cancer bone metastasis. *British journal of pharmacology*, 178(9), pp.1923-1935.

Masood, S., (2016). Neoadjuvant chemotherapy in breast cancers. *Women's Health*, 12(5), pp.480-491.

Masoud, G.N. and Li, W., (2015). HIF-1α pathway: role, regulation and intervention for cancer therapy. *Acta Pharmaceutica Sinica* B, 5(5), pp.378-389.

Massagué, J. and Obenauf, A.C., (2016). Metastatic colonization by circulating tumour cells. *Nature*, 529(7586), pp.298-306.

McCoy, E.M., Hong, H., Pruitt, H.C. and Feng, X., (2013). IL-11 produced by breast cancer cells augments osteoclastogenesis by sustaining the pool of osteoclast progenitor cells. *Bmc Cancer,* 13, pp.1-11.

Meednu, N., Zhang, H., Owen, T., Sun, W., Wang, V., Cistrone, C., Rangel-Moreno, J., Xing, L. and Anolik, J.H., (2016). Production of RANKL by memory B cells: a link between B cells and bone erosion in rheumatoid arthritis. *Arthritis & rheumatology*, *68*(4), pp.805-816.

Merdad, A., Karim, S., Schulten, H.J., Dallol, A., Buhmeida, A., Al-Thubaity, F., Gari, M.A., Chaudhary, A.G., Abuzenadah, A.M. and Al-Qahtani, M.H., (2014). Expression of matrix

metalloproteinases (MMPs) in primary human breast cancer: MMP-9 as a potential biomarker for cancer invasion and metastasis. *Anticancer research*, *34*(3), pp.1355-1366.

Mishra, A., Shiozawa, Y., Pienta, K.J. and Taichman, R.S., (2011). Homing of cancer cells to the bone. *Cancer Microenvironment*, 4, pp.221-235.

Mitri, Z.I., Abuhadra, N., Goodyear, S.M., Hobbs, E.A., Kaempf, A., Thompson, A.M. and Moulder, S.L., (2022). Impact of TP53 mutations in Triple Negative Breast Cancer. NPJ *Precision Oncology*, 6(1), p.64.

Moore-Smith, L. and Pasche, B., (2011). TGFBR1 signaling and breast cancer. *Journal of mammary gland biology and neoplasia,* 16, pp.89-95.

Ng, N., Purshouse, K., Foskolou, I.P., Olcina, M.M. and Hammond, E.M., (2018). Challenges to DNA replication in hypoxic conditions. *The FEBS journal*, 285(9), pp.1563-1571.

Ni, X., Xia, T., Zhao, Y., Zhou, W., Wu, N., Liu, X., Ding, Q., Zha, X., Sha, J. and Wang, S., (2014). Downregulation of miR-106b induced breast cancer cell invasion and motility in association with overexpression of matrix metalloproteinase 2. *Cancer science*, 105(1), pp.18-25.

Nishimura, K., Shindo, S., Movila, A., Kayal, R., Abdullah, A., Savitri, I.J., Ikeda, A., Yamaguchi, T., Howait, M., Al-Dharrab, A. and Mira, A., (2016). TRAP-positive osteoclast precursors mediate ROS/NO-dependent bactericidal activity via TLR4. *Free Radical Biology and Medicine*, 97, pp.330-341.

Nutter, F., Holen, I., Brown, H.K., Cross, S.S., Evans, C.A., Walker, M., Coleman, R.E., Westbrook, J.A., Selby, P.J., Brown, J.E. and Ottewell, P.D., (2014). Different molecular profiles are associated with breast cancer cell homing compared with colonisation of bone: evidence using a novel bone-seeking cell line. *Endocrine-related cancer*, 21(2), pp.327-341.

Parker, B. and Sukumar, S., (2003). Distant metastasis in breast cancer: molecular mechanisms and therapeutic targets. *Cancer biology & therapy*, 2(1), pp.13-22.

Patel, A., Unni, N. and Peng, Y., (2020). The changing paradigm for the treatment of HER2-positive breast cancer. *Cancers*, 12(8), p.2081.

Pellegrino, B., Hlavata, Z., Migali, C., De Silva, P., Aiello, M., Willard-Gallo, K., Musolino, A. and Solinas, C., (2021). Luminal breast cancer: Risk of recurrence and tumor-associated immune suppression. *Molecular Diagnosis & Therapy*, 25(4), pp.409-424.

Pereira, B., Chin, S.F., Rueda, O.M., Vollan, H.K.M., Provenzano, E., Bardwell, H.A., Pugh, M., Jones, L., Russell, R., Sammut, S.J. and Tsui, D.W., (2016). The somatic mutation profiles of 2,433 breast cancers refine their genomic and transcriptomic landscapes. *Nature communications*, 7(1), p.11479.

Petrova, V., Annicchiarico-Petruzzelli, M., Melino, G. and Amelio, I., (2018). The hypoxic tumour microenvironment. *Oncogenesis*, 7(1), p.10

Piccart, M., Procter, M., Fumagalli, D., de Azambuja, E., Clark, E., Ewer, M.S., Restuccia, E., Jerusalem, G., Dent, S., Reaby, L. and Bonnefoi, H., (2021). Adjuvant pertuzumab and trastuzumab in early HER2-positive breast cancer in the APHINITY trial: 6 years' follow-up. *Journal of Clinical Oncology*, 39(13), pp.1448-1457.

Pivetta, E., Scapolan, M., Pecolo, M., Wassermann, B., Abu-Rumeileh, I., Balestreri, L., Borsatti, E., Tripodo, C., Colombatti, A. and Spessotto, P., (2011). MMP-13 stimulates osteoclast differentiation and activation in tumour breast bone metastases. *Breast Cancer Research*, *13*, pp.1-15.

Poujade, F.A., (2016). THE UNIVERSITY OF HULL.

Poujade, F.A., Mannion, A., Brittain, N., Theodosi, A., Beeby, E., Leszczynska, K.B., Hammond, E.M., Greenman, J., Cawthorne, C. and Pires, I.M., (2018). WSB-1 regulates the metastatic potential of hormone receptor negative breast cancer. *British journal of cancer*, *118*(9), pp.1229-1237.

Price, T.T., Burness, M.L., Sivan, A., Warner, M.J., Cheng, R., Lee, C.H., Olivere, L., Comatas, K., Magnani, J., Kim Lyerly, H. and Cheng, Q., (2016). Dormant breast cancer micrometastases reside in specific bone marrow niches that regulate their transit to and from bone. *Science translational medicine*, 8(340), pp.340ra73-340ra73.

Rachner, T.D., Kasimir-Bauer, S., Göbel, A., Erdmann, K., Hoffmann, O., Browne, A., Wimberger, P., Rauner, M., Hofbauer, L.C., Kimmig, R. and Bittner, A.K., (2019). Prognostic

value of RANKL/OPG serum levels and disseminated tumor cells in nonmetastatic breast cancer. *Clinical Cancer Research*, *25*(4), pp.1369-1378.

Rad, F.R., Ajdary, S., Omranipour, R., Alimohammadian, M.H. and Hassan, Z.M., (2015). Comparative analysis of CD4+ and CD8+ T cells in tumor tissues, lymph nodes and the peripheral blood from patients with breast cancer. *Iranian biomedical journal*, *19*(1), p.35.

Raja, R., Kale, S., Thorat, D., Soundararajan, G., Lohite, K., Mane, A., Karnik, S. and Kundu, G.C., (2014). Hypoxia-driven osteopontin contributes to breast tumor growth through modulation of HIF1α-mediated VEGF-dependent angiogenesis. *Oncogene*, 33(16), pp.2053-2064.

Rana, D., Kumar, A. and Sharma, S., (2018). Endothelial progenitor cells as molecular targets in vascular senescence and repair. *Current stem cell research & therapy*, 13(6), pp.438-446.

Rankin, E.B., Nam, J.M. and Giaccia, A.J., (2016). Hypoxia: signaling the metastatic cascade. *Trends in cancer*, 2(6), pp.295-304.

Razorenova, O.V., Finger, E.C., Colavitti, R., Chernikova, S.B., Boiko, A.D., Chan, C.K., Krieg, A., Bedogni, B., LaGory, E., Weissman, I.L. and Broome-Powell, M., (2011). VHL loss in renal cell carcinoma leads to up-regulation of CUB domain-containing protein 1 to stimulate PKCδ-driven migration. *Proceedings of the National Academy of Sciences*, 108(5), pp.1931-1936.

Recio-Saucedo, A., Gerty, S., Foster, C., Eccles, D. and Cutress, R.I., (2016). Information requirements of young women with breast cancer treated with mastectomy or breast conserving surgery: A systematic review. *The Breast*, 25, pp.1-13.

Reed, E., Simmonds, P., Haviland, J. and Corner, J., (2012). Quality of life and experience of care in women with metastatic breast cancer: a cross-sectional survey. *Journal of pain and symptom management,* 43(4), pp.747-758.

Regev, O., Kizner, M., Roncato, F., Dadiani, M., Saini, M., Castro-Giner, F., Yajuk, O., Kozlovski, S., Levi, N., Addadi, Y. and Golani, O., (2022). ICAM-1 on breast cancer cells suppresses lung metastasis but is dispensable for tumor growth and killing by cytotoxic T cells. *Frontiers in immunology*, *13*, p.849701.

Regulski, M., Regulska, K., Prukała, W., Piotrowska, H., Stanisz, B. and Murias, M., (2016). COX-2 inhibitors: a novel strategy in the management of breast cancer. *Drug discovery today*, 21(4), pp.598-615.

Rhodes, D.R. and Chinnaiyan, A.M., (2005). Integrative analysis of the cancer transcriptome. *Nature genetics*, 37(Suppl 6), pp.S31-S37.

Ristimäki, A., Sivula, A., Lundin, J., Lundin, M., Salminen, T., Haglund, C., Joensuu, H. and Isola, J., (2002). Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer research*, 62(3), pp.632-635.

Saadatmand, S., Bretveld, R., Siesling, S. and Tilanus-Linthorst, M.M., (2015). Influence of tumour stage at breast cancer detection on survi val in modern times: population based study in 173 797 patients. *Bmj*, 351.

Salamanna, F., Borsari, V., Contartese, D., Costa, V., Giavaresi, G. and Fini, M., (2019). What is the role of interleukins in breast cancer bone metastases? A systematic review of preclinical and clinical evidence. *Cancers*, 11(12), p.2018.

Sameti, P., Tohidast, M., Amini, M., Bahojb Mahdavi, S.Z., Najafi, S. and Mokhtarzadeh, A., (2023). The emerging role of MicroRNA-182 in tumorigenesis; a promising therapeutic target. Cancer Cell International, 23(*1*), p.134.

Schmid, P., Cortes, J., Pusztai, L., McArthur, H., Kümmel, S., Bergh, J., Denkert, C., Park, Y.H., Hui, R., Harbeck, N. and Takahashi, M., (2020). Pembrolizumab for early triple-negative breast cancer. *New England Journal of Medicine*, 382(9), pp.810-821.

Schneider, C.A., Rasband, W.S. and Eliceiri, K.W., (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature methods*, 9(7), pp.671-675.

Sethi, N., Dai, X., Winter, C.G. and Kang, Y., (2011). Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells. *Cancer cell,* 19(2), pp.192-205.

Shah, L., Latif, A., Williams, K.J., Mancuso, E. and Tirella, A., (2023). Invasion and secondary site colonization as a function of in vitro primary tumor matrix stiffness: breast to bone metastasis. *Advanced Healthcare Materials*, 12(3), p.2201898.

Sharma, B., Nannuru, K.C., Saxena, S., Varney, M.L. and Singh, R.K., (2019). CXCR2: A novel mediator of mammary tumor bone metastasis. *International journal of molecular sciences*, 20(5), p.1237.

Shichrur, K., Feinberg-Gorenshtein, G., Luria, D., Ash, S., Yaniv, I. and Avigad, S., (2014). Potential role of WSB1 isoforms in growth and survival of neuroblastoma cells. *Pediatric Research*, 75(4), pp.482-486.

Singh, B., Berry, J.A., Shoher, A., Ayers, G.D., Wei, C. and Lucci, A., (2007). COX-2 involvement in breast cancer metastasis to bone. *Oncogene*, *26*(26), pp.3789-3796.

Singh, D., Arora, R., Kaur, P., Singh, B., Mannan, R. and Arora, S., (2017). Overexpression of hypoxia-inducible factor and metabolic pathways: possible targets of cancer. *Cell & bioscience*, 7(1), pp.1-9.

Slay, E.E., Meldrum, F.C., Pensabene, V. and Amer, M.H., (2021). Embracing mechanobiology in next generation organ-on-A-chip models of bone metastasis. *Frontiers in Medical Technology,* 3, p.722501.

Soki, F.N., Park, S.I. and McCauley, L.K., (2012). The multifaceted actions of PTHrP in skeletal metastasis. *Future oncology*, 8(7), pp.803-817.

Song, X., Wei, C. and Li, X., (2022). The signaling pathways associated with breast cancer bone metastasis. *Frontiers in Oncology*, 12, p.855609.

Song, Y., Barry, W.T., Seah, D.S., Tung, N.M., Garber, J.E. and Lin, N.U., (2020). Patterns of recurrence and metastasis in BRCA1/BRCA2-associated breast cancers. *Cancer*, 126(2), pp.271-280.

Spadazzi, C., Mercatali, L., Esposito, M., Wei, Y., Liverani, C., De Vita, A., Miserocchi, G., Carretta, E., Zanoni, M., Cocchi, C. and Bongiovanni, A., (2021). Trefoil factor-1 upregulation in estrogen-receptor positive breast cancer correlates with an increased risk of bone metastasis. *Bone*, 144, p.115775.

Stark, A.M., Anuszkiewicz, B., Mentlein, R., Yoneda, T., Mehdorn, H.M. and Held-Feindt, J., (2007). Differential expression of matrix metalloproteinases in brain-and bone-seeking clones of metastatic MDA-MB-231 breast cancer cells. *Journal of neuro-oncology*, *81*, pp.39-48.

Sun, X. and Kaufman, P.D., (2018). Ki-67: more than a proliferation marker. *Chromosoma*, *127*, pp.175-186.

Sun, Y., Liu, Y., Ma, X. and Hu, H., (2021). The influence of cell cycle regulation on chemotherapy. *International journal of molecular sciences*, 22(13), p.6923.

Suzuki, E., Ochiai-Shino, H., Aoki, H., Onodera, S., Saito, A., Saito, A. and Azuma, T., (2014). Akt activation is required for TGF-β1-induced osteoblast differentiation of MC3T3-E1 pre-osteoblasts. *PLoS One*, 9(12), p.e112566.

Swami, S., Zhu, H., Nisco, A., Kimura, T., Kim, M.J., Nair, V. and Wu, J.Y., (2023). Parathyroid hormone 1 receptor signaling mediates breast cancer metastasis to bone in mice. *JCI insight*, *8*(5).

Tahara, R.K., Brewer, T.M., Theriault, R.L. and Ueno, N.T., (2019). Bone metastasis of breast cancer. *Breast Cancer Metastasis and Drug Resistance: Challenges and Progress*, pp.105-129.

Tang, K., Zhu, L., Chen, J., Wang, D., Zeng, L., Chen, C., Tang, L., Zhou, L., Wei, K., Zhou, Y. and Lv, J., (2021). Hypoxia promotes breast cancer cell growth by activating a glycogen metabolic program. *Cancer research*, 81(19), pp.4949-4963.

Tang, G., Liu, J., Gao, X., Tang, W., Chen, J., Wu, M., Lv, Z., Zhang, Y., Cai, Y. and Qi, L., (2024). circWSB1 promotes tumor progression in ccRCC via circWSB1/miR-182-5p/WSB1 axis. International Journal of Biological Macromolecules, 256, p.128338.

Taniguchi, K. and Karin, M., (2018). NF-κB, inflammation, immunity and cancer: coming of age. Nature Reviews Immunology, 18(*5*), pp.309-324.

Tesfamariam, Y., Jakob, T., Wöckel, A., Adams, A., Weigl, A., Monsef, I., Kuhr, K. and Skoetz, N., (2019). Adjuvant bisphosphonates or RANK-ligand inhibitors for patients with breast cancer and bone metastases: a systematic review and network meta-analysis. *Critical Reviews in Oncology/Hematology*, 137, pp.1-8.

Thomas, R., Al-Khadairi, G. and Decock, J., (2021). Immune checkpoint inhibitors in triple negative breast cancer treatment: promising future prospects. *Frontiers in oncology*, 10, p.600573.

Tian, Z., Yu, C., Zhang, W., Wu, K.L., Wang, C., Gupta, R., Xu, Z., Wu, L., Chen, Y., Zhang, X.H.F. and Xiao, H., (2022). Bone-Specific Enhancement of Antibody Therapy for Breast Cancer Metastasis to Bone. *ACS Central Science*, 8(3), pp.312-321.

Tong, Y., Li, Q.G., Xing, T.Y., Zhang, M., Zhang, J.J. and Xia, Q., (2013). HIF1 regulates WSB-1 expression to promote hypoxia-induced chemoresistance in hepatocellular carcinoma cells. *FEBS letters*, 587(16), pp.2530-2535.

Trivedi, T., Pagnotti, G.M., Guise, T.A. and Mohammad, K.S., (2021). The role of TGF-β in bone metastases. *Biomolecules*, 11(11), p.1643.

Tripathi, M., Colige, A. and Deroanne, C.F., (2023). The Dual Function of RhoGDI2 in Immunity and Cancer. International Journal of Molecular Sciences, 24(*4*), p.4015.

Tsukamoto, H., Takeuchi, S., Kubota, K., Kobayashi, Y., Kozakai, S., Ukai, I., Shichiku, A., Okubo, M., Numasaki, M., Kanemitsu, Y. and Matsumoto, Y., (2018). Lipopolysaccharide (LPS)-binding protein stimulates CD14-dependent Toll-like receptor 4 internalization and LPS-induced TBK1–IKKε–IRF3 axis activation. *Journal of Biological Chemistry*, *293*(26), pp.10186-10201.

Tulotta, C. and Ottewell, P., (2018). The role of IL-1B in breast cancer bone metastasis. *Endocrine-related cancer*, *25*(7), pp.R421-R434.

Tulotta, C., Lefley, D.V., Freeman, K., Gregory, W.M., Hanby, A.M., Heath, P.R., Nutter, F., Wilkinson, J.M., Spicer-Hadlington, A.R., Liu, X. and Bradbury, S.M., (2019). Endogenous production of IL1B by breast cancer cells drives metastasis and colonization of the bone microenvironment. *Clinical Cancer Research*, *25*(9), pp.2769-2782.

van Barele, M., Rieborn, A., Heemskerk-Gerritsen, B.A., Obdeijn, I.M., Koppert, L.B., Loo, C.E., Tollenaar, R.A., Ausems, M.G., van de Beek, I., Berger, L.P. and de Boer, M., (2022). Survival of BRCA1/BRCA2-associated pT1 breast cancer patients, a cohort study. *Breast Cancer Research and Treatment,* 194(1), pp.159-170.

Vasiliauskas, D., Hancock, S., and Stern, C.D. (1999). SWiP-1: novel SOCS box containing WD-protein regulated by signalling centres and by Shh during development. Mech. Dev. 82, 79–94

Végran, F., Boidot, R., Michiels, C., Sonveaux, P. and Feron, O., (2011). Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-κB/IL-8 pathway that drives tumor angiogenesis. *Cancer research*, 71(7), pp.2550-2560.

Venetis, K., Piciotti, R., Sajjadi, E., Invernizzi, M., Morganti, S., Criscitiello, C. and Fusco, N., (2021). Breast cancer with bone metastasis: molecular insights and clinical management. *Cells*, 10(6), p.1377.

Vik, A., Brodin, E.E., Mathiesen, E.B., Brox, J., Jørgensen, L., Njølstad, I., Brækkan, S.K. and Hansen, J.B., (2015). Serum osteoprotegerin and future risk of cancer and cancer-related mortality in the general population: the Tromsø study. *European journal of epidemiology*, *30*, pp.219-230.

Vishal, M., Swetha, R., Thejaswini, G., Arumugam, B. and Selvamurugan, N., (2017). Role of Runx2 in breast cancer-mediated bone metastasis. *International journal of biological macromolecules*, 99, pp.608-614.

Waks, A.G. and Winer, E.P., (2019). Breast cancer treatment: a review. Jama, 321(3), 288-300.

Wang, H. and Mao, X., (2020). Evaluation of the efficacy of neoadjuvant chemotherapy for breast cancer. *Drug design, development and therapy*, pp.2423-2433.

Wang, L., Zhang, S. and Wang, X., 2021. The metabolic mechanisms of breast cancer metastasis. Frontiers in Oncology, 10, p.602416.

Wang, N., He, Y.L., Pang, L.J., Zou, H., Liu, C.X., Zhao, J., Hu, J.M., Zhang, W.J., Qi, Y. and Li, F., (2015). Down-regulated E-cadherin expression is associated with poor five-year overall survival in bone and soft tissue sarcoma: results of a meta-analysis. *PloS one*, 10(3), p.e0121448.

Wang, R., Zhu, Y., Liu, X., Liao, X., He, J. and Niu, L., (2019). The Clinicopathological features and survival outcomes of patients with different metastatic sites in stage IV breast cancer. *BMC cancer*, 19(1), pp.1-12.

Wang, Y., Lei, R., Zhuang, X., Zhang, N., Pan, H., Li, G., Hu, J., Pan, X., Tao, Q., Fu, D. and Xiao, J., (2014). DLC1-dependent parathyroid hormone–like hormone inhibition suppresses breast cancer bone metastasis. *The Journal of clinical investigation*, 124(4), pp.1646-1659.

Wang, Y., Liu, Y., Huang, Z., Chen, X. and Zhang, B., (2022). The roles of osteoprotegerin in cancer, far beyond a bone player. *Cell Death Discovery*, 8(1), p.252.

Wazir, U., Ahmed, M.H., Bridger, J.M., Harvey, A., Jiang, W.G., Sharma, A.K. and Mokbel, K., (2013). The clinicopathological significance of lamin A/C, lamin B1 and lamin B receptor mRNA expression in human breast cancer. *Cellular & molecular biology letters*, 18, pp.595-611.

Wei, H., Xu, Z., Chen, L., Wei, Q., Huang, Z., Liu, G., Li, W., Wang, J., Tang, Q. and Pu, J., (2022). Long non-coding RNA PAARH promotes hepatocellular carcinoma progression and angiogenesis via upregulating HOTTIP and activating HIF-1α/VEGF signaling. *Cell death & disease*, 13(2), p.102.

Weichhaus, M., Chung, S.T.M. and Connelly, L., (2015). Osteoprotegerin in breast cancer: beyond bone remodeling. *Molecular cancer*, 14, pp.1-7.

Weinberg, R.A. and Hanahan, D., 2000. The hallmarks of cancer. Cell, 100(1), pp.57-70.

Wells, A., Yates, C. and Shepard, C.R., (2008). E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clinical & experimental metastasis*, 25, pp.621-628.

Weng, Y., Pan, C., Shen, Z., Chen, S., Xu, L., Dong, X. and Chen, J., (2022). Identification of potential WSB1 inhibitors by AlphaFold modeling, virtual screening, and molecular dynamics simulation studies. *Evidence-Based Complementary and Alternative Medicine*, 2022.

Wengner, A.M., Scholz, A. and Haendler, B., (2020). Targeting DNA damage response in prostate and breast cancer. *International journal of molecular sciences*, 21(21), p.8273.

Widschwendter, M., Burnell, M., Fraser, L., Rosenthal, A.N., Philpott, S., Reisel, D., Dubeau, L., Cline, M., Pan, Y., Yi, P.C. and Evans, D.G., (2015). Osteoprotegerin (OPG), the endogenous inhibitor of receptor activator of NF-κB ligand (RANKL), is dysregulated in BRCA mutation carriers. *EBioMedicine*, *2*(10), pp.1331-1339.

Wiechmann, L., Sampson, M., Stempel, M., Jacks, L.M., Patil, S.M., King, T. and Morrow, M., (2009). Presenting features of breast cancer differ by molecular subtype. *Annals of surgical oncology,* 16(10), 2705-2710.

Wright, L.E., Ottewell, P.D., Rucci, N., Peyruchaud, O., Pagnotti, G.M., Chiechi, A., Buijs, J.T. and Sterling, J.A., (2016). Murine models of breast cancer bone metastasis. *BoneKEy reports*, 5.

Wu, C., Chen, M., Sun, Z., Ye, Y., Han, X., Qin, Y. and Liu, S., (2019). Wenshen Zhuanggu formula mitigates breast cancer bone metastasis through the signaling crosstalk among the Jagged1/Notch, TGF-β and IL-6 signaling pathways. *Journal of ethnopharmacology*, 232, pp.145-154.

Wu, M.Y., Li, C.J., Yiang, G.T., Cheng, Y.L., Tsai, A.P.Y., Hou, Y.T., Ho, Y.C., Hou, M.F. and Chu, P.Y., (2018). Molecular regulation of bone metastasis pathogenesis. *Cellular Physiology and Biochemistry*, *46*(4), 1423-1438.

Wu, Q., Li, J., Zhu, S., Wu, J., Chen, C., Liu, Q., Wei, W., Zhang, Y. and Sun, S., (2017). Breast cancer subtypes predict the preferential site of distant metastases: a SEER based study. *Oncotarget*, 8(17), p.27990.

Xu, H., Han, H. and Tian, G., (2020). High expression of WSB1 is associated with poor prognosis in hepatocellular carcinoma and affects epithelial-mesenchymal transition. J. *BUON official J. Balkan Union Oncol*, 25(4), p.1890.

Xu, J., Acharya, S., Sahin, O., Zhang, Q., Saito, Y., Yao, J., Wang, H., Li, P., Zhang, L., Lowery, F.J. and Kuo, W.L., (2015). 14-3-3ζ turns TGF-β's function from tumor suppressor to metastasis promoter in breast cancer by contextual changes of Smad partners from p53 to Gli2. *Cancer cell*, *27*(2), pp.177-192.

Xu, J., Liu, J., Gan, Y., Dai, K., Zhao, J., Huang, M., Huang, Y., Zhuang, Y. and Zhang, X., (2020). High-dose TGF-β1 impairs mesenchymal stem cell–mediated bone regeneration via Bmp2 inhibition. *Journal of Bone and Mineral Research*, 35(1), pp.167-180.

Xu, Y., Gao, X.D., Lee, J.H., Huang, H., Tan, H., Ahn, J., Reinke, L.M., Peter, M.E., Feng, Y., Gius, D. and Siziopikou, K.P., (2014). Cell type-restricted activity of hnRNPM promotes breast cancer metastasis via regulating alternative splicing. *Genes & development*, 28(11), pp.1191-1203.

Yamada, K.M., (2000). Fibronectin peptides in cell migration and wound repair. *The Journal of clinical investigation*, 105(11), pp.1507-1509.

Yan, S., Wang, Y., Liu, P., Chen, A., Chen, M., Yao, D., Xu, X., Wang, L. and Huang, X., (2016). Baicalin attenuates hypoxia-induced pulmonary arterial hypertension to improve hypoxic cor pulmonale by reducing the activity of the p38 MAPK signaling pathway and MMP-9. *Evidence-Based Complementary and Alternative Medicine*, *2016*.

Yang, M., Xu, S.P. and Ao, Q.L., (2013). Expression of fatty acid synthase and its association with HER2 in invasive ductal carcinoma of breast. Zhonghua Bing li xue za zhi. *Chinese Journal of Patholo*gy, 42(4), pp.257-261.

Yang, X., Ren, H., Sun, Y., Shao, Y., Zhang, L., Li, H., Zhang, X., Yang, X., Yu, W. and Fu, J., 2017. Prognostic significance of CD4/CD8 ratio in patients with breast cancer. Int J Clin Exp Pathol, 10(4), pp.4787-93.

Yang, Y., Gao, L., Chen, J., Xiao, W., Liu, R. and Kan, H., (2022). Lamin B1 is a potential therapeutic target and prognostic biomarker for hepatocellular carcinoma. *Bioengineered*, 13(4), pp.9211-9231.

Yao, Y., Chu, Y., Xu, B., Hu, Q. and Song, Q., (2019). Risk factors for distant metastasis of patients with primary triple-negative breast cancer. *Bioscience Reports*, 39(6), p.BSR20190288.

Ye, Y., Yang, Z. and Lei, J., (2019). Stochastic telomere shortening and the route to limitless replicative potential. *Journal of Computational Biology*, 26(4), pp.350-363.

Yu, H., Pardoll, D. and Jove, R., (2009). STATs in cancer inflammation and immunity: a leading role for STAT3. *Nature reviews cancer*, 9(11), pp.798-809.

Yuan, X., Qian, N., Ling, S., Li, Y., Sun, W., Li, J., Du, R., Zhong, G., Liu, C., Yu, G. and Cao, D., (2021). Breast cancer exosomes contribute to pre-metastatic niche formation and promote bone metastasis of tumor cells. *Theranostics*, 11(3), p.1429.

Zhang, J., Guo, F., Li, C., Wang, Y., Wang, J., Sun, F., Zhou, Y., Ma, F., Zhang, B. and Qian, H., (2023). Loss of TTC17 promotes breast cancer metastasis through RAP1/CDC42 signaling and sensitizes it to rapamycin and paclitaxel. *Cell & Bioscience*, 13(1), p.50.

Zhang, L., Qu, J., Qi, Y., Duan, Y., Huang, Y.W., Zhou, Z., Li, P., Yao, J., Huang, B., Zhang, S. and Yu, D., (2022). EZH2 engages TGFβ signaling to promote breast cancer bone metastasis via integrin β1-FAK activation. *Nature communications*, *13*(1), p.2543.

Zhang, M., Zhang, Y.Y., Chen, Y., Wang, J., Wang, Q. and Lu, H., (2021). TGF-β signaling and resistance to cancer therapy. *Frontiers in cell and developmental biology*, 9, p.786728.

Zhang, T., Suo, C., Zheng, C. and Zhang, H., (2019). Hypoxia and metabolism in metastasis. *Hypoxia and cancer metastasis*, pp.87-95.

Zhang, X., Huang, S., Guo, J., Zhou, L., You, L., Zhang, T. and Zhao, Y., (2016). Insights into the distinct roles of MMP-11 in tumor biology and future therapeutics. *International journal of oncology,* 48(5), pp.1783-1793.

Zhang, X., Karim, M., Hasan, M.M., Hooper, J., Wahab, R., Roy, S. and Al-Hilal, T.A., (2022). Cancer-on-a-Chip: Models for studying metastasis. *Cancers*, 14(3), p.648.

Zhang, X., Yang, L., Chien, S. and Lv, Y., (2018). Suspension state promotes metastasis of breast cancer cells by up-regulating cyclooxygenase-2. *Theranostics*, *8*(14), p.3722.

Zhang, Y., He, W. and Zhang, S., (2019). Seeking for correlative genes and signaling pathways with bone metastasis from breast cancer by integrated analysis. *Frontiers in oncology*, 9, p.138.

Zhang, J., Yu, Y., Zou, X., Du, Y., Liang, Q., Gong, M., He, Y., Luo, J., Wu, D., Jiang, X. and Sinclair, M., (2024). WSB1/2 target chromatin-bound lysine-methylated RelA for proteasomal degradation and NF-κB termination. Nucleic acids research, 52(*9*), pp.4969-4984.

Zhao, S., Zuo, W.J., Shao, Z.M. and Jiang, Y.Z., (2020). Molecular subtypes and precision treatment of triple-negative breast cancer. *Annals of translational medicine*, 8(7).

Zhou, J., Down, J.M., George, C.N., Murphy, J., Lefley, D.V., Tulotta, C., Alsharif, M.A., Leach, M. and Ottewell, P.D., (2022). Novel methods of targeting IL-1 signalling for the treatment of breast cancer bone metastasis. *Cancers*, *14*(19), p.4816.

Zhou, J., Tulotta, C. and Ottewell, P.D., (2022). IL-1β in breast cancer bone metastasis. *Expert Reviews in Molecular Medicine*, 24, p.e11.

Zhou, Q., Xu, J., Xu, Y., Sun, S. and Chen, J., (2023). Role of ICAM1 in tumor immunity and prognosis of triple-negative breast cancer. *Frontiers in Immunology*, 14.