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Role of the Tumour Microenvironment in Breast Cancer Bone Metastasis

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“Science means constantly walking a tightrope between blind faith and curiosity; between expertise and creativity; between bias and openness; between experience and epiphany; between ambition and passion; and between arrogance and conviction - in short, between an old today and a new tomorrow”

- Henrich Rohrer

Dedication

I dedicate my thesis to my dad, Alexander Martin Graham, who fought courageously and relentlessly in his war against cancer, and to my mum, Elma Graham, who stood beside him in every battle. Whilst like too many others he lost his war, he inspired me to continue the fight against the disease through research.

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Just over four years have passed since I first stepped into the lab ready to embark on my PhD. I have thoroughly enjoyed my time as a PhD student, and I am grateful for all I have learned and the opportunities that it has given me. As it comes to an end, I wish to offer my appreciation for all of those who have supported me throughout the journey.

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Finally, I wish to thank my family for their support not only during my PhD, but throughout all of my education. My parents have provided support and encouragement, and shown love and enthusiasm for what I do from the start. They raised my aspirations making me believe I could achieve whatever I set out to do, and it is therefore to you I owe my success.

Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

Nicola Graham

Abstract

Cancer metastasis remains the largest challenge in oncology accounting for over 90% of cancer-associated deaths. The bone is the most prevalent site of metastasis in many cancers, including breast cancer where 70% of patients with advanced disease exhibit bone metastasis. With a current lack of curative treatments, patients with metastasis to the bone have a 5-year survival rate of approximately 15%. Recent focus on the tumour microenvironment (TME) has now identified an important role in cancer development. By establishing interactions with the tumour cells, the TME promotes tumour growth, metastasis and treatment resistance. However, much of the research into the TME has been performed in primary tumour models and research into the role of the TME in bone metastasis is limited.

Using *in vivo* experimental models of breast cancer bone metastasis complemented with *in vitro* assays, we investigated the role of the TME throughout breast cancer bone metastasis development, including early stage seeding, metastatic growth and during chemotherapy treatment. We first demonstrated that macrophages play a significant role during tumour cell seeding to the bone where the depletion of macrophages during tumour cell seeding resulted in reduced tumour cell number and delayed metastatic growth. Novel intravital imaging and imaging of cleared tissues were also developed and are currently being used to visualise the seeding process in real time. We next investigated the role of the TME in the growth of breast cancer bone metastasis. An increased infiltration of mesenchymal stem cells (MSCs) was observed in the bone metastasis, which when depleted using Nestin-GFP-CreER^{T2}-iDTR mice resulted in significant decrease in bone metastatic growth in comparison to non-depleted controls. *In vitro* proliferation assays demonstrated that direct contact between bone metastatic breast cancer cells and MSCs promotes tumour cell growth. Furthermore, preliminary data demonstrates that the co-operation between MSCs and macrophages within the TME may also be important in tumour growth. Lastly, we investigated the effect of chemotherapy treatment on breast cancer bone metastasis. Whilst bone metastasis appears to be resistant to doxorubicin, paclitaxel treatment results in increased growth, indicating paclitaxel-induced metastatic growth. The doxorubicin resistance and paclitaxel-induced growth are both dependent on the microenvironment in the bone, therefore flow cytometry, immunostaining techniques and cell depletion models were performed to

characterise the component of the TME responsible for the effects of chemotherapy treatment.

Collectively, in this project we demonstrate the role of the TME during the development of breast cancer bone metastasis including the macrophages during tumour cell seeding and MSCs during bone metastasis growth. The TME also plays a pivotal role in chemotherapy resistance and induced growth of bone metastasis. The identification of the molecular mechanisms involved in these processes may offer insight into potential novel therapeutic targets to overcome breast cancer bone metastasis.

Lay Summary

One in eight women will be diagnosed with breast cancer in their lifetime. Over the last few decades improvements in ways to screen, diagnose and treat breast cancer means more patients are surviving the disease. However, those who are diagnosed with late stage breast cancer, defined as that which has already spread or metastasized to other parts of the body, have a poor chance of survival. The most common place for breast cancer to spread to is the bone. Once cancer has spread here, only 15% of patients will survive after 5 years. Those who do survive past this time remain incurable and can only be treated with palliative care to treat symptoms. There is therefore a need to investigate new ways we can treat bone metastasis.

Cancers have been described as 'wounds that do not heal'. This is because cancers are not only made up of cancer cells but also other types of cells involved in wound healing. These include immune cells, stromal cells, signalling molecules and a molecular scaffold that supports the cells. Together, this is called the tumour microenvironment (TME). In this project we look at how the TME contributes to the development of bone metastasis of breast cancer.

When cancer cells first arrive in the bone, they must go to a specific location in the bone, called a 'niche' to help them survive, similar to how a seed will only thrive in the right soil. We show a type of immune cell called a macrophage is involved in promoting breast cancer cells to seed to the bone. When macrophages are removed from mice, the number of cancer cells that seed to the bone is reduced and the growth is delayed significantly. Using live microscopic imaging of the bone through a specially implanted window on the mouse leg, we hope to show exactly how these macrophages help the cancer cells arrive in the bone.

We next investigated the cells of the TME that help the cancer cells to grow in the bone. Another type of cells in the bone is called mesenchymal stem cells (MSCs). We show using immunofluorescent staining that these cells are increased in bone metastasis and infiltrate the cancerous region. We next used genetically modified mice to remove the MSCs during growth of bone metastasis. This reduces the growth of the bone metastasis so that the cancer is almost eradicated, which confirms that the MSCs promote growth of bone metastasis. Our next steps will be to investigate the molecules that the mesenchymal stem cells produce and act on the tumour cells to make them grow.

Finally we investigated the effect of chemotherapy on bone metastasis. When we treat bone metastasis in mice with a type of chemotherapy called doxorubicin, there is no difference in growth compared to untreated metastasis, suggesting the bone metastasis is resistant to the chemotherapy. However, when the mice were given another type of chemotherapy called paclitaxel, the size of the bone metastasis, in fact, increases. This phenomenon is known as chemotherapy-induced metastasis. We show that this effect is due to the cells and environment in the bone, and we are therefore currently investigating the exact cell responsible for the chemotherapy resistance and chemotherapy-induced metastasis.

Together, we have shown that different cells in the TME play a role at each stage of bone metastasis development. The next steps in the research would be to identify the molecules and signalling between the cancer cells and the cells in the TME, and to develop new drugs to target these. This may help find a potential therapy to treat this currently incurable disease.

Abbreviations

α MEM	Alpha Minimum Essential Media
α SMA	Alpha smooth muscle actin
μ l	Microlitre
μ m	Micrometre
2D	Two-dimensional
3D	Three dimensional
3DISCO	Three-dimensional imaging of solvent-cleared organs
AF	Alexa Fluor
ALP	Alkaline phosphatase
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
BA:BB	Benzyl alcohol: benzyl benzoate
BB:PEG	Benzyl benzoate:polyethylene glycol
BCL2	B cell lymphoma 2
BCL _{XL}	B cell lymphoma extra large
BCRP	Breast cancer resistant protein
BLI	Bioluminescence imaging
BM-MSC	Bone marrow mesenchymal stem cell
BMP	Bone morphogenetic protein
BoMAM	Bone metastasis associated macrophage
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
BSP	Bone sialoprotein
C1q	Complement component 1q
CAF	Cancer associated fibroblast
CC3	Cleaved caspase 3
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CEMIP	Cell migration inducing hyaluronidase 1
Chi3L1/2	Chitinase 3-like 1/2
CM	Conditioned media
COX1/2	Cyclooxygenase 1/2
CSC	Cancer stem cell
CSF1	Colony stimulating factor 1
CSF1R	Colony-stimulating factor 1 Receptor
CTC	Circulatory tumour cell
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAMP	Danger-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma in situ
DKK1	Dickkopf-related protein 1
Dm ³	Cubic decimetre
DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DT	Diphtheria toxin
DTC	Disseminated tumour cell
DTR	Diphtheria toxin receptor
ECM	Extracellular matrix

EDTA	Ethylene Diamine Tetra Acetic Acid
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast activated protein
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FoxO1	Forkhead box O1
FSP	Fibroblast specific protein
GAS6	Growth arrest-specific 6
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBMSC	Human bone marrow mesenchymal stem cell
HBSS	Hank's Balanced Salt Solution
HDGF	Hepatocyte-derived growth factor
HGF	Hepatocyte growth factors
HIF1 α	Hypoxia inducible factor 1 alpha
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
IC	Intracardiac
IDC	Invasive ductal carcinoma
IFN γ	Interferon-gamma
IGF	Insulin-like growth factor
IIA	Intraliac artery
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IV	Intravenous
JNK	c-Jun N-terminal kinase
Lepr	Leptin Receptor
LOX	Lysyl oxidase
Ly6C	Ly6 complex locus C
Ly6G	Lymphocyte antigen 6 complex locus G6D
MAFIA	Macrophage Fas-Induced Apoptosis
MAM	Metastasis associated macrophage
MAPK	Mitogen-activated protein kinase
MCAM	Melanoma cell adhesion molecule
mCh	mCherry
MCL	Induced myeloid leukemia cell differentiation protein
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid derived suppressor cell
MDT	Macrophage deprivation therapy
MFP	Mammary fat pad
MHC I/II	Major histocompatibility complex I/II
miRNA	microRNA
MMP	Matrix metalloproteinase
MMTV-PyMT	Mouse mammary tumour virus-polyoma middle tumour antigen
MRP1	Multidrug resistance protein 1
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF	Normal fibroblast
NF- κ B	Nuclear factor kappa B

NG2	Neurone-Glial antigen 2
NGS	Normal goat serum
NK cell	Natural killer cell
NSCLC	Non-small cell lung carcinoma
OB	Osteoblast
OCT	Optical Cutting Temperature compound
OD	Optical density
OG	Oral gavage
OPG	Osteoprotegerin
OSM	Oncostatin M
OPT	Optical projection tomography
P/S	Penicillin/Streptomycin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1
PDGF	Platelet derived growth factor
PDGF-BB	Platelet derived growth factor - BB
PDGFR α/β	Platelet derived growth factor receptor alpha
PDL1	Programmed death-ligand 1
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PGE ₂	Prostaglandin E ₂
PHD2	prolyl hydroxylase domain 2
PI3K	Phosphoinositide 3-kinase
PKM2	Pyruvate kinase M2
PR	Progesterone receptor
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PTX	Paclitaxel
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROI	Region of interest
ROS	Reactive oxygen species
SCF	Stem cell factor
SEM	Standard error of the mean
siRNA	Small interfering RNA
SR-A	Scavenger receptor A
STAT	Signal transducer and activator of transcription
SUMO	small ubiquitin-like modifier
TAM	Tumour-associated macrophage
TGF β	Transforming growth factor beta
Th1/2	Type 1/2helper T cell
Tim4	T-cell membrane protein 4
TLR	Toll-like receptor
TME	Tumour microenvironment
TMEM	Tumour microenvironment of metastasis
TNBC	Triple negative breast cancer
TNF α	Tumour necrosis factor alpha
TPO	Thrombopoietin
Treg	Regulatory T cell
uPA	Urokinase plasminogen activator
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial factor
VWF	von Willebrand factor
Zhx2	zinc fingers and homeoboxes 2

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Chapter 1 Introduction

1.1 Breast cancer

Breast cancer is the most common female malignancy with one in eight women developing the disease at some point throughout their life¹. In 2018, approximately 2.08 million women were diagnosed with breast cancer worldwide, accounting for 24.2% of total new cancer cases in females and 10-15% of total cancers diagnosed. Recent data shows that approximately 55,200 women are diagnosed with breast cancer annually in the UK alone, which is expected to increase². Despite the increasing incidence of breast cancer, the overall mortality has declined and survival rates have improved, doubling over the last 40 years from 40% to approximately 78%. This is attributed to development in breast cancer screening and diagnosis, and improved treatments including chemotherapy, surgery and radiotherapy as well as novel targeted therapies³.

Nevertheless, breast cancer is also a major contributor to the cancer-related deaths worldwide and is the 4th most common cause of cancer-associated deaths. There are currently 11,400 breast cancer associated deaths in the UK each year, accounting for 7% of total cancer deaths and 15% of cancer-associated deaths in women¹. The mortality rate is relatively low compared to other more aggressive cancer types, and is particularly true for patients who receive early diagnosis with approximately a 5-year survival rate 98% for women diagnosed in stage 1. However, the survival rate decreases with stage, and those diagnosed with late metastatic disease face a poor prognosis with a 5-year survival of about 15-25% (figure 1.1.1)⁴.

Metastatic disease is detected in 6-10% of patients at the time of initial diagnosis, defined as *de novo* breast cancer, and it is estimated that 20-30% of women with earlier stages will go on to develop recurrent or metastatic breast cancer⁴. The recurrence of metastasis significantly impairs the clinical outcome and patients exhibit a poor prognosis with a median survival duration of 2-3 years reducing 5-year survival to 15%⁵. Late stage cancer is currently incurable with limited treatment options available to manage symptoms and constrain further progression using palliative care.⁶

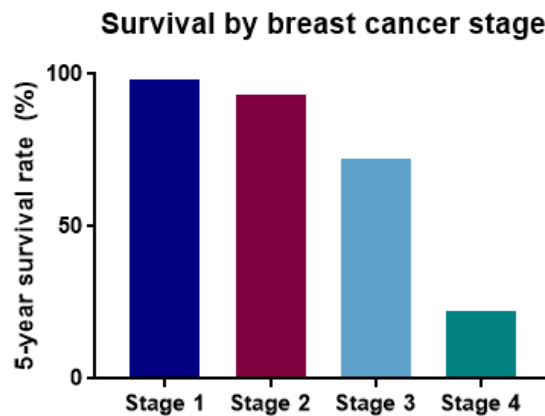


Figure 1.1.1: Overall 5-year survival rate by breast cancer stage. Localised stage 1 disease 98-99%; regional stage 2 disease 86-90%; stage 3 disease 70%; metastatic stage 4 disease 15-25%.

Breast cancer most commonly originates in the breast ducts with 70% of cases arising here. The earliest stage of breast cancer is stage 0, known as ductal carcinoma in situ (DCIS), and is a non-invasive cancer where the cancerous cells remain in the lining of the breast milk duct. In the successive stages, known as invasive ductal carcinoma (IDC), the cancer cells invade the surrounding tissue and beyond. Other rarer types of invasive breast cancer include invasive lobular carcinoma that arises from the lobules in the breast and accounts for 15% of cases, and inflammatory breast cancer, which is much less common and arises from cancer cells developing in the lymph vessels in the skin resulting in blockage and subsequently an inflammatory response. Invasive ductal carcinoma is also categorised into stages based on the tumour size, lymph node involvement and spread to other organs. Stage 1 is early stage cancer confined to the breast, or lymph nodes closest to the breast, and is small (<2cm) in size.

Approximately 80% of breast cancer patients are diagnosed at this stage. Stage 2 is still an early stage breast cancer and is defined as cancer 2-5cm that involves 1-3 axillary or breast bone lymph nodes. Stage 3, also known as regionally advanced breast cancer, involves more lymph nodes (4-9) and has spread to the chest wall or skin of the breast. Stage 4, known as metastatic breast cancer, is the most advanced stage of breast cancer when the disease has spread to other organs in the body.

Breast cancer commonly metastasises to the brain, lung, liver and bone, with the bone being the most frequent with approximately 70% of patients with advanced disease exhibiting bone metastasis⁷.

1.2 Breast cancer bone metastasis

Although patients diagnosed with early stage diseases have a high 5-year survival rate of 90-98%, patients who develop secondary metastasis to the bone exhibit a poor prognosis with a 5-year survival rate of 15-25%.

The bone is the most common site of metastasis in many cancers, including breast, prostate, myeloma and lung cancer^{1,8,9}. Breast and prostate cancer display a particular organ tropism towards the bone with 65-75% and 90% of patients, respectively, exhibiting evidence of bone metastasis at autopsy¹⁰. In breast cancer almost 40% of late stage patients exhibit bone-only metastasis whereas approximately 33% will develop bone metastasis in combination with metastasis at other sites^{6,7,11}. This is significantly more frequent than to other sites (table 1.2.1). In a recent study, 3.28%, of newly diagnosed patients presented with bone metastasis, compared to 1.52%, 1.20%, and 0.35% for the lung, liver, and brain metastases, respectively¹². Moreover, up to 13.6% of patients diagnosed with stage I-III breast cancer will develop bone metastasis¹³. First relapse occurs between 11-20 months after initial diagnosis, which is significantly quicker than to other sites such as the lung that take on average 56 months to recur. Subsequently, novel skeletal events occur on average every 3-6 months^{8,14}.

The prognosis for patients with bone metastasis is considerably better compared to recurrence and metastasis in the visceral sites. It is often considered as an indolent disease and patients with bone metastasis display a better survival duration than patients with other metastases including lung, liver and brain (table 1.2.1). However, the bone is the site of the most significant burden and these patients exhibit significant morbidity.

Metastatic site	% patients with late stage disease	Median 5-year survival (months)
Bone	39.8	37.2-44.4
Lung	10.94	14.4-58.5
Liver	7.34	15-36.7
Brain	1.51	6-7.35
Other	7.34	13.2
Multiple	33.07	14.4

Table 1.2.1: Prevalence and survival of each breast cancer metastasis by metastatic site^{6, 15}.

1.2.1 Morbidity associated with bone metastasis

Patients with bone metastasis experience substantial morbidity, greater than other metastatic site, and includes extreme pain, increased risk of fracture and hypercalcemia, which together significantly impair quality of life^{14,16}.

Bone metastasis is the most prevalent cause of cancer-related pain, and is the major complication of bone metastasis. Whilst the pathological reasons for the pain remain to be fully elucidated, it is thought to involve the osteolytic effects, the production of growth factors and cytokines that mediate an inflammatory response, and aberrant stimulation of the nerves. Mechanical pain can also occur where the pressure of the tumour within the bone causes significant pain, and is exacerbated by the osteolysis. Pain often becomes severe and fails to be alleviated by commonly used analgesics, therefore requires careful pain management^{8,17}.

Bone metastasis, particularly osteolytic lesions, results in the destruction of the bone (discussed later). Due to the osteolysis during bone metastasis, hypercalcemia is a common complication and is present in 80% of cases. It is particularly prevalent in patients of breast cancers. Elevated calcium levels occur as a result of increased bone resorption, which releases calcium into the serum. Parathyroid hormone-related protein (PTHrP) is also released from the both and is elevated in approximately two-thirds of bone metastasis patients with hypercalcaemia. Renal impairment also contributes to hypercalcemia in some cases, and often due to the action of PTHrP¹⁴. If left untreated, increased serum calcium can result in lethal renal failure and cardiac arrhythmias as well as other symptoms such as constipation, polyuria and extreme fatigue. Furthermore, hypercalcaemia is associated with liver metastasis, which it is thought to promote by unknown mechanisms^{8,17}.

The metastatic-induced destruction of the bone also reduces the strength and load-bearing capability. This consequently results in initial microfractures, which are extremely painful, and eventually pathological fractures occur in 10-30% of patients predominantly in the femur and vertebrae^{8,17,18}. This can be devastating to the patient as they significantly reduce the quality of life. Furthermore, the inflammatory response and healing may in fact promote further bone metastasis. Fractures are particularly problematic in the ribs and vertebrae where it causes collapse and potential risk to the lung and spinal column, respectively. One of the most critical complications of fractures during bone metastasis is spinal cord compression and is observed in 20-30% of

breast cancer patients. Initially this causes pain that precedes neurological complications including weakness, numbness and paralysis. This outcome not only significantly impedes life quality, but is also associated with a poor prognosis and a reduced median survival of 4 months¹⁷.

1.2.2 Treatments of breast cancer bone metastasis

The standard of care for breast cancer patients traditionally includes surgery to physically remove the tumour as well as lymph nodes, and chemotherapy and radiotherapy that exert cytotoxic effects on the tumours cells. Both chemotherapy and radiotherapy is required in an adjuvant setting if there is a risk of cancer relapse. It can also be provided if in a neoadjuvant setting to initially reduce the tumour size before surgery. Hormone, or endocrine, therapies are now used frequently in the clinic for oestrogen receptor (ER) positive breast cancer in an adjuvant setting after surgery. They include tamoxifen and aromatase inhibitors, which antagonist ER and inhibit oestrogen production, respectively. More recently, novel targeted therapies have been developed to specifically target specific subtypes of breast cancer. Herceptin positive breast cancers are now targeted with a treatment called trastuzumab or Pertuzumab, which are anti-Herceptin antibodies that antagonises the Herceptin receptor and inhibits the downstream signalling pathway leading to attenuated cell growth. Another targeted therapy recently developed is Olaparib, which is used in BRCA mutated cancers. As a PARP inhibitor, this drug induces synthetic lethality resulting in the cancer cell death.

Breast cancers with different histopathological and biological features exhibit distinct behaviours that lead to different treatment responses that must be considered during treatment. The treatment of breast cancer is therefore dependent on the clinical stages as well as the molecular subtype, defined by the PAM50 characterisation (table 1.2.2)¹⁹. Whilst luminal cancers respond well to hormone therapy, they respond poorly to chemotherapy. In contrast Herceptin⁺ breast cancer responds well to anthracycline and taxane-based chemotherapy and exhibits significantly higher pathological responses than luminal cancer. The basal subtype, known as triple negative breast cancer (TNBC), cannot be treated with endocrine or targeted therapies, resulting in treatment options limited to surgery, radiotherapy and chemotherapy to which the TNBC is often resistant²⁰.

Subtype	Expression status	Outcome	Prevalence
Luminal A	ER+ PR+ Her2- Ki67-	Good	23.7%
Luminal B	ER+ PR+ Her2- Ki67+	Intermediate	38.8%
	ER+ PR+ Her2+ Ki67+	Poor	14%
Her2 ⁺ overexpression	ER- PR- Her2+	Poor	11.2%
Basal	ER- PR- Her2-	Poor	12.3%
Normal-like	ER+ PR+ Her2- Ki67-	Intermediate	7.8%

Table 1.2.2: Breast cancer molecular subtypes defined by the PAM50 gene signature. (ER oestrogen receptor, PR progesterone receptor; Her2 herceptin2)^{19,20}.

Despite these significant advances in the treatment of primary breast cancer, the treatment options for patients with bone metastasis remains limited due to resistance to most therapies. There have been several developments in endocrine therapies, including optimisation of the aromatase inhibitors, letrozole, anastrozole, and exemestane, which show promise in treating breast cancer bone metastasis. However, these are only suitable for oestrogen positive breast cancers and limited responses are observed²¹. A palliative approach is therefore used with the primary aim of slowing progression and alleviating symptoms to maintain quality of life. Bone metastasis is often treated as a chronic condition using several treatments including chemotherapy and radiotherapy to restrict further tumour. However, there is currently very little knowledge regarding the optimal management of bone metastasis⁹.

Radiotherapy is also used to relieve pain via mechanisms that are poorly understood. It can be administered using three forms of treatment: local-field radiation is the conventional treatment used to target specific localised sites of bone metastasis whereas wide-field radiation also known as systemic radiation is used for widespread bone metastases. Alternatively, radionuclide therapy uses radioisotopes that emit ionising radiation and are administered systemically to target widespread metastases. Whilst radiation alleviates pain in 80-90% of patients, new non-irradiated lesions/events occur and result in significant pain^{17,18,21}.

Due to the prominent role of osteoclast activation and osteolysis in bone metastasis, osteoclast inhibitors, such as bisphosphonates, have become an integral component of palliative care for bone metastasis. Bisphosphonates are analogues of pyrophosphate, which is a natural inhibitor of bone demineralization. They localise to resorbing bone by binding to exposed bone mineral around resorbing osteoclasts where the function to both inhibit osteoclasts and induce apoptosis of them. This prevents osteoclastogenesis and bone resorption⁸. By disrupting the osteolysis, bisphosphonates prevent bone loss thus manages or overcomes the complications and

symptoms associated with bone metastasis. This includes management of pain as it has been demonstrated that bisphosphonates significantly reduce metastatic bone pain independent of other interventions^{18,22–24}. They are also the drug of choice in preventing hypercalcemia with 70-90% of patients obtaining normal calcium levels^{8,21,23}. It has also recently been demonstrated that bisphosphonates exhibit anti-tumour activity possibly related to tumour angiogenesis, and macrophage infiltration²⁵. Clodronate and pamidronate were traditionally the two most commonly used bisphosphonates, however, novel forms have been developed, including zoledronate and ibandronate, which exhibit a greater potency and thus is more efficient in managing metastasis. The bisphosphonates are generally well tolerated with minimal adverse effects including mild fever, fatigue and nausea^{8,21}. Similarly, denosumab is a human monoclonal antibody that prevents the development of osteoclasts and is often utilised in bone metastasis patients to help prevent or delay fractures. It is well tolerated and therefore is often indicated for patients with other severe complications such as impaired renal function, or when bisphosphonates display resistance⁸.

Surgical interventions are often required for bone metastasis and are indicated for fractures of long bones and hip joints. Prophylactic surgery is often performed to reinforce the bones using the insertion of rods to prevent the fractures. However, careful consideration is required for surgery due to the short-term prognosis of patients with bone metastasis and the risk that patients will not be able to recover from the procedure further limiting quality of life^{9,17}.

Whilst palliative treatments such as anti-osteolytic bisphosphonates are available to improve symptoms and lessen the morbidity associated with bone metastasis, these do not significantly enhance survival. Bone metastases are often resistant to anti-tumour treatments and therefore there remains no cure.

1.3 Pathogenesis of bone metastasis

The most common site for bone metastases is the axial skeleton including the spine, ribs and pelvic bones, as well as the femurs. This pattern correlates with areas of the red marrow, which is enriched in vasculature, immune cells, mesenchymal cells, and extracellular matrix. Together this mediates the recruitment and development of bone metastasis¹⁴. The tumour cells reach the bone via the vessels feeding the marrow and once established, bone metastasis disrupts the normal bone structure and homeostasis. It is therefore important to describe the normal structure, cellular

composition and function of the bone in order to understand the pathophysiology of bone metastasis.

1.3.1 Structure and function of bone

The skeleton is one of the largest organs in the body. It has several physical functions including providing protection to vital organs and supporting the body together with other components of the musculoskeletal system. The bone also plays important systemic roles including providing a niche for haematopoiesis, and storage of minerals, particularly calcium, fat and growth factors, which are released or absorbed to maintain systemic levels^{26–28}.

The bone is an organised structure and is not uniformly solid. The outer layer of the bone, known as the cortical or compact bone, is the densest compartment of the bone serving as the strength and weight-bearing properties. It consists of a mineralized matrix that is arranged into organised structures called osteons, and composed of predominantly type I collagen, which is crosslinked to provide the tensile strength. The bone also contains salts including calcium, phosphate and hydroxyapatite, of which the latter provides compressive strength and load-bearing properties. Trabecular bone is located at the proximal and distal ends of long bones in the vicinity to the joints. In contrast to the cortical bone, the trabecular bone is less dense and a more spongy, porous structure. Both the cortical and trabecular bone is lined by a thin vascular membrane, known as the periosteum and endosteum on the outside and inside, respectively. A medullary cavity containing the bone marrow is located within the centre of the bone, and is predominantly found in the femur, ribs, vertebrae and pelvic bone. Flat bones, such as the skull, only have a thin layer of marrow. The marrow contains cells including the haematopoietic stem cells (HSCs) and well as adipose tissue^{26–28}.

1.3.1.1 Cellular components of the bone

The bone matrix is also embedded with a variety of cell types that contribute to both the structure and maintenance of the bone. This includes mesenchymal stromal cells, osteoclasts and osteoblasts as well as other cell types such as endothelial cells and adipocytes that will not be discussed.

1.3.1.1.1 Mesenchymal stem cells and pericytes

Mesenchymal stem cells (MSCs) are a rare, but vital population of cells within the bone. They were first described in 1974 by Friedenstein and colleagues, who identified a population of bone marrow cells that formed fibroblastic colonies derived from single cells^{29,30}. However, it was not until 1991 that Caplan defined the population as MSCs due to their ability to self-renew and differentiate to give rise to bone, cartilage and mesodermal tissues^{31,32}. Since first being identified over 40 years ago, the true lineage and identification has remained controversial due to the lack of a specific marker to define MSCs. This has created difficulty in finding the exact identify of MSCs, particularly *in vivo*, and studies involving MSC-like cells have used different defining characteristics to identify the cells. These identification issues of MSCs can impede research, and is further complicated by their multiple tissue origins. Minimal criteria have therefore been determined to define MSCs *in vitro* and must be met when used in research to help achieve standardisation. By this, MSCs from the bone marrow are defined as plastic adherent cells with a long, thin, spindle-like morphology, and must express CD105, CD90 and CD73, but lack expression of immune marker CD45, endothelial marker CD31, haematopoietic marker CD34, monocyte markers CD11b or CD14, B cell markers CD79a or CD19, and HLA-DR. MSCs cultured should display the ability to self-renew and exhibit a tri-lineage differentiation potential into osteoblasts, chondrocytes, and adipocytes. It should be noted, however, that they are also capable of differentiating into other cell lineages (figure 1.3.1)³³.

MSCs are distributed throughout many tissues including bone, adipose tissue and fetal umbilical cord and are key players in many homeostatic processes. They are required for tissue maintenance, regeneration and repair of connective tissues including bone, cartilage and adipose tissue by differentiating into osteoblasts, chondrocytes and adipocytes, respectively. MSCs are also recruited to wounds during repair where they produce matrix proteins, initiate apoptosis and secrete growth factors, cytokines and chemokines that promote recruitment of other cells such as immune cells. Within the bone, MSCs are a rare population making up about 0.001-0.01% of total cells. They not only contribute to bone turnover by differentiating into the bone-producing osteoblasts, but also provide a perivascular and endosteal compartment that maintains the haematopoietic stem cells (HSCs), known as the HSC niche^{32,34,35}. Several MSC-like populations exist within the bone each defined by different markers, including CXCL12-producing (CAR) cells, nestin⁺ MSCs, and leptin receptor (lepr)⁺ MSCs^{36,37}. CD146⁺

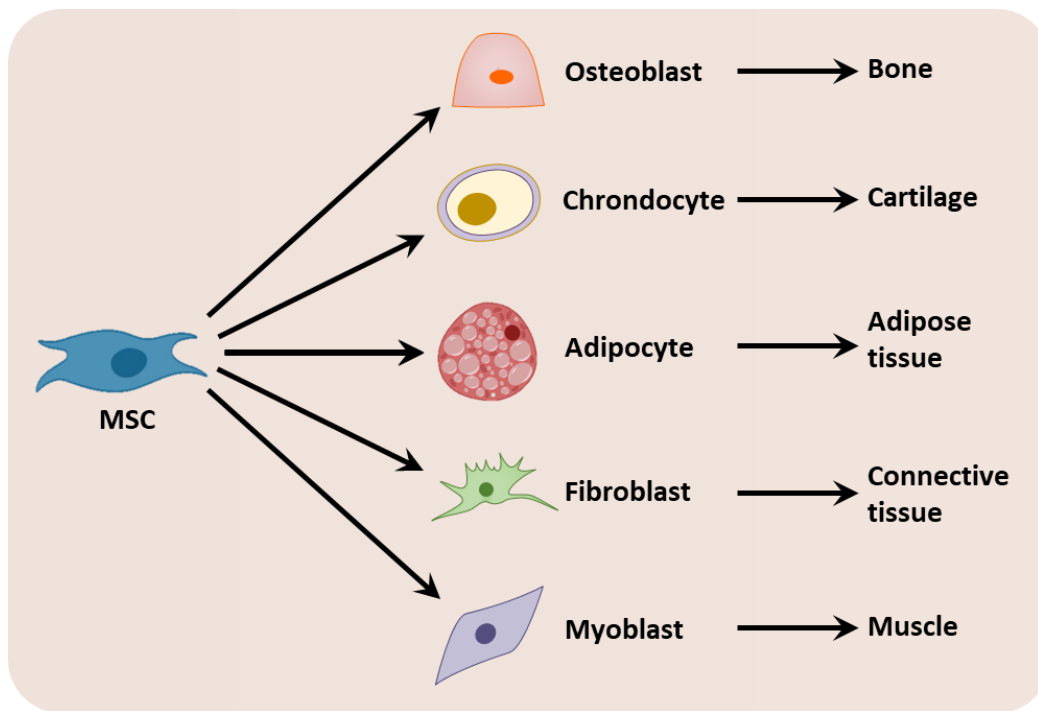


Figure 1.3.1: Lineage-specific differentiation of MSCs.

MSCs have also recently been identified and are often associated with the vasculature³⁸. Moreover, a population of cells similar to MSCs, defined as pericytes, lie on the outer layer of blood vessels adjacent to the endothelial cells where they maintain blood vessel integrity. Pericytes share characteristics to those that define MSCs *in vitro* including the differentiation into osteoblasts, chondrocytes and adipocytes, as well as smooth muscle cells and skeletal muscle cells. Whilst they were classically defined by the expression of neurone-glia antigen 2 (NG2) and platelet derived growth factor receptor alpha (PDGFR β), it was recently demonstrated that NG2⁺ PDGFR β ⁺ pericyte-like perivascular cells expressed α -smooth muscle actin (α SMA) and CD146 as well as other lineage markers defining MSCs³⁵. This suggests that classical pericytes may be similar to perivascular MSCs, and may in fact be a subpopulation of perivascular MSCs. It appears that not all pericytes are MSCs nor are all MSCs likely to be pericytes, however, the extent of the overlap remains unclear. The studies investigating the identity of the MSCs and MSC-like cells based on the expression of above markers will be discussed later as well as their functions within the HSC niche.

1.3.1.1.2 *Osteoclast, osteoblasts and osteocytes*

Two of the most important cells within the bone are osteoblasts and osteoclasts, which are responsible for bone remodeling through the deposition of new bone and resorption of old bone, respectively.

Osteoblasts line the bone both at the periosteum and the endosteum, and are specialized, differentiated cells of the mesenchymal stem cell lineage. The differentiation and maturation of MSCs to osteoblasts is regulated through a sequence of transcription factor expression. Initially Wnt10b promotes MSCs differentiation to an osteo/chondrocyte progenitor, whilst suppressing adipocyte differentiation. Runx2 then induces commitment to the osteoblast lineage producing an osteoprogenitor, which is further differentiated into a pre-osteoblast and subsequently a mature osteoblast by osterix, also known as SP7, that acts downstream of Runx2. These transcription factors are activated by bone morphogenic proteins (BMPs), transforming growth factor beta (TGF β), parathyroid hormone (PTH) and fibroblast growth factor (FGF), which are released during bone resorption. Mature osteoblasts secrete bone matrix proteins, including alkaline phosphatase (ALP), collagen 1, osteopontin, osteocalcin and osteonectin during bone deposition³⁹. Osteoblasts finally give rise to another population of bone cells known as osteocytes, which become embedded in the matrix. Here they form an extensive cellular network that is thought to mediate skeletal structure by acting as mechano-sensors and regulating the activity of osteoblasts and osteoclasts in response to changes in load-bearing⁴⁰.

Osteoclasts are the professional bone-resorbing cells of the bone, which are derived from the myeloid lineage through a process known as osteoclastogenesis. HSCs give rise to a myeloid progenitor under the control of transcription factor PU.1. Subsequently signalling pathways acting in a sequential manner regulate osteoclast differentiation. Firstly macrophage colony-stimulating factor (M-CSF) induces commitment to the osteoclast lineage giving rise to osteoclast progenitors. These subsequently express Receptor Activator of Nuclear Factor κ B (RANK) which interacts with RANK ligand (RANKL) on the surface of osteoblasts to complete differentiation to mature osteoclasts^{39,41}.

The osteoblasts are integral regulators of osteoclastogenesis through the RANK/RANKL. RANKL is expressed on the surface of osteoblasts where it activates RANK and therefore osteoclastogenesis. Osteoblasts also secrete osteoprotegerin

(OPG), which is a secreted protein similar to RANK, and functions to sequester and inhibit RANKL. This prevents osteoclastogenesis and therefore osteoclast-mediated bone resorption. The balance between OPG and RANKL expression regulates the activity of both osteoclasts and osteoblasts and therefore mediates bone turn over and remodelling (figure 1.3.2a)⁴¹.

1.3.1.1.3 Osteomacs

Bone resident macrophages, defined as osteomacs, are also located at bone remodelling sites including the endosteum and periosteum where co-locate with osteoblasts forming a distinctive canopy structure covering over 75% of mature osteoblasts⁴². The depletion of osteomacs using the Macrophage Fas-Induced Apoptosis (MAFIA) mouse model results in a complete loss of the osteoblasts at the bone-forming surface in the calvaria. The removal of osteomacs from calvarial culture also significantly decreased osteocalcin production and osteoblast mineralization *in vitro*⁴². Whilst this role of macrophages was initially demonstrated in calvaria⁴² and intramembranous repair⁴³, a similar role was revealed in the long bones during endochondral ossification repair by Vi *et al.*⁴⁴. Macrophage depletion in MAFIA mice model resulted in retarded bone growth and maintenance of bone density observed as an osteoporosis phenotype. Furthermore, there was a 60% reduction in bone marrow MSCs and a decreased ability to differentiate to osteoblasts⁴⁴. The role of macrophages in osteogenic differentiation of MSCs to osteoblast differentiation was confirmed using *in vitro* assays. Co-culture of M2-like macrophages with MSCs results in increased differentiation to osteoblasts indicated by an increase in ALP activity and osteogenic genes, including Runx2, collagen 1, osteocalcin, BMPs and bone sialoprotein 11 (BSP11)^{42,44–51}. Together, these studies demonstrate that osteomacs are important in the maintenance of the osteoblast population of the bone, and are required for osteoblast-mediated bone deposition during remodelling.

1.3.2 Disruption of the bone turnover during metastasis

Bone metastasis interferes with both the structure and the function of the bone. This includes disruption to the bone turnover and remodelling, as well as exploitation of the HSC niche.

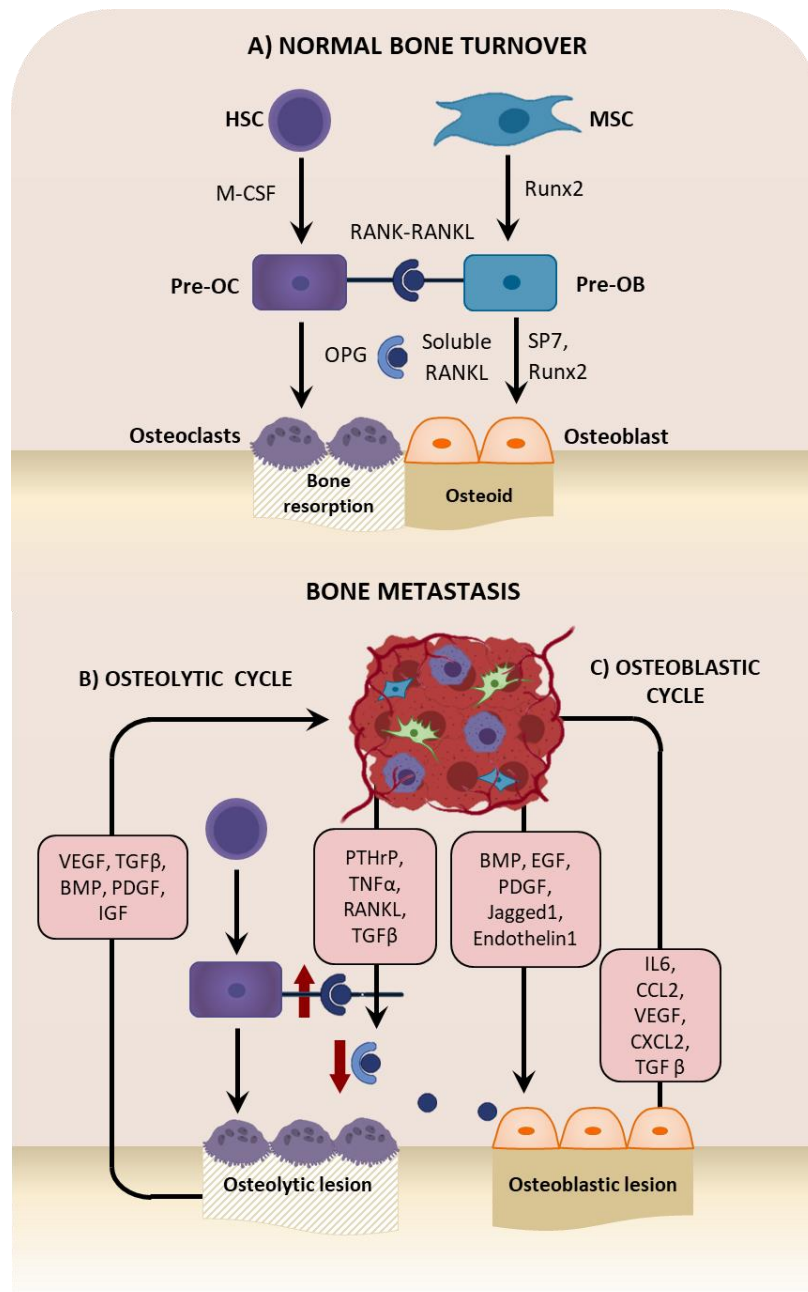


Figure 1.3.2: Vicious cycle of bone metastasis **A)** Normal bone homeostasis is controlled by the balance of osteoblast and osteoclast activity. Haematopoietic stem cells (HSCs) differentiate into osteoclasts known as osteoclastogenesis. Osteoclasts secrete proteolytic enzymes such as cathepsin K and MMP that induce bone resorption. MSCs differentiate into osteoblasts under the control of transcription factors, Runx2 and osterix/SP7. Osteoblasts produce osteoid that matures to mineralized bone. Osteoblasts also mature into osteocytes within the bone. Osteoblasts also regulate bone resorption via RANKL production, which binds to RANK on the osteoclasts to activate osteoclastogenesis, and via osteoprotegerin, which inhibits RANKL therefore osteoclastogenesis. **B)** Osteolytic bone metastasis disrupt bone homeostasis by releasing growth factors and cytokines, which activate RANKL and inhibit OPG resulting in increased osteoclastogenesis therefore bone resorption. This releases growth factors that promote TC growth, creating a vicious cycle resulting in a significant osteolytic lesion within the bone. **C)** Osteoblastic bone metastasis exhibit an additional paracrine loop involving factors released from the tumour cells that induce osteoblast expansion and activation, and subsequent release of growth factors that act upon the tumour cells⁵²⁻⁵⁴.

1.3.2.1 Bone homeostasis, remodelling and turnover in normal bone

The bone is a dynamic tissue that is continuously remodelled. Bone turnover and maintenance is dependent on a delicate balance between the activity of the bone-forming osteoblasts and bone-resorbing osteoclasts, and is a highly orchestrated multicellular process consisting of 4 stages. The activation phase consists of recruitment and activation of osteoclasts to the site. Stimuli such as microfractures or alterations in mechanical loading are sensed by the osteocytes and induce the secretion of soluble factors such as Insulin-like growth factor (IGF1), tumour necrosis factor alpha (TNF α), PTH and IL6. These promote the bone lining cells to upregulate RANKL, which interacts with RANK on the surface of pre-osteoclasts in resulting in differentiation to osteoclast. During the resorption phase, osteoclasts destroy the organic component of the bone via the release of degrading enzymes, including cathepsin K and matrix metalloproteinase 9 (MMP9), and dissolve the inorganic components via the acidification of the matrix. The osteoclasts subsequently undergo apoptosis and macrophage-like cells are recruited to remove debris, defined as the reverse phase. Finally, the formation phase occurs where growth factors released from the bone matrix during resorption, including BMPs, FGFs and TGF β , activate and recruit osteoblasts to the resorbed area. Here they deposit new bone matrix called osteoid composed of predominantly collagen fibres as well osteopontin and osteocalcin that subsequently mineralises⁵⁵. The deposition of matrix from osteoblasts is initially weak and unorganised, known as woven bone, and is rearranged into sheets of organised collagen exhibiting a strong composition defined as lamellar bone.

Remodelling persists throughout life and serves many functions. In the long term, remodelling replaces old and damaged bone such as ischaemic or microfractured regions in order to maintain the structural integrity and strength. It also functions to regulate the calcium homeostasis. The bone acts as a calcium reservoir and remodelling regulate the uptake or release of calcium in certain conditions such as vitamin D deficiency, and thus maintains normal serum calcium levels⁵⁵.

1.3.2.2 Effect of bone metastasis on bone turnover

Once bone metastatic cells have established as a metastatic lesion in the bone, they disrupt the bone turn over and remodelling by skewing the balance between osteoblast and osteoclast activity. This is classified as osteolytic, osteoblastic or mixed, in accordance with the interference on the bone turnover (figure 1.3.2b, figure 1.3.2c),

and is associated with breast, prostate and myeloma bone metastasis, respectively. However, these more than likely represent the extremes of the process and both coexist within the same lesions. For example, 15-20% of breast cancer patients with bone metastasis exhibit osteoblastic lesions in addition to osteolytic lesions.

Osteolytic metastasis is mediated predominantly by the release of TGF β and PTHrP from the tumour cells. Several studies demonstrate that PTHrP is overexpressed in breast cancer and stimulate osteoclast activation⁵⁶. Release of these molecules suppresses the activity of OPG from osteoblasts, and therefore permits RANK/RANKL interaction resulting in osteoclastogenesis and bone resorption. In turn, the degradation of the bone releases growth factors from the matrix, including TGF β , IGF, platelet derived growth factor (PDGF) and BMP, that act upon the tumour to induce further growth. Subsequently this induces a self-sustaining positive feedback loop, defined as the 'vicious cycle' of bone metastasis^{17,54}. Several components of this paracrine loop have been therapeutically targeted, including RANKL, OPG and TGF β to inhibit bone metastasis recurrence⁵⁴. Osteolytic metastatic cancer cells also exploit osteoclasts by inducing the secretion of MMP9 and cathepsin K to elicit the pathological osteoclast activity and excessive bone resorption⁴¹.

Osteoblastic metastasis is characterised by the deposition of new bone and is characteristic of prostate cancer bone metastasis. Whilst the mechanisms of osteoblastic metastasis are less defined, it has been demonstrated that growth cycle observed in osteolytic lesions is accompanied by the release of growth factors from tumour cells, such as epidermal growth factor (EGF), BMP, PDGF and PTH, which act upon the osteoblasts to induce activation and expansion. In turn, osteoblast-derived growth factors, including vascular endothelial factor (VEGF), interleukin 6 (IL6), CCL2, and CXCL12 are released, which promotes tumour progression, initiating an additional cycle^{8,54,57}. There is also evidence that identifies other paracrine interactions between the tumour cells and osteoblasts that mediate bone metastatic growth, including TGF β , jagged1, endothelin-1, integrins, and annexin II⁵⁴.

1.3.3 Utilisation of the HSC niche during bone metastasis

The bone is responsible for haematopoiesis, which refers to the differentiation of HSCs to give rise to the myeloid and lymphoid compartment of the immune system, as well as erythroid lineages and osteoclasts within the bone. This process is regulated by the HSC niches within the bone that facilitate the maintenance and differentiation of the

HSCs^{28,58}. There is now substantial evidence to demonstrate that bone metastatic cells utilise the same molecular interactions that exist between the HSCs and niche cells, and disrupt the haematopoietic function. Within the niche, the stromal cells provide the cancer cells with survival factors and protect them from anti-tumour treatments, including chemotherapy. For this reason, bone metastasis has been referred to as a 'molecular parasite' that exploits the resources from the niche environment⁵⁹. The existence of this pre-formed HSC niche may also account for the favourable tropism of metastasis to the bone as there is no dependence on the initial establishment of a pre-metastatic⁶⁰. In order to understand how the tumour cells hi-jack this system, it is important to first understand the cellular components of the HSC niche and the interactions that exist between the stromal cells within the niche and HSCs.

1.3.3.1 The HSC niche

The concept of a HSC niche was first proposed by Schofield in 1978 referring to a regulatory unit that maintained the HSC population. Since then, research has identified two vital HSC niches within the bone: the endosteal niche comprising of predominantly osteoblasts that promote HSC homing and quiescence; and the perivascular niche that consists predominantly of mesenchymal-like cells and regulates HSC proliferation and mobilization (figure 1.3.3)⁶¹. Other cells are also involved in both niches including sinusoidal endothelium, perivascular stromal cells, adipocytes and immune cells. Within the niches, HSCs establish interactions with the niche cells to mediate adhesion. Both the perivascular and endosteal niche influence the behaviour of the HSCs by regulation of different signalling cascades and together synergistically mediate self-renewal, proliferation and differentiation.

The endosteal niche is comprised of the osteoblasts that cover the endosteum, as well as other niche cells including the sinusoidal endothelium and perivascular stromal cells. Two types of osteoblast exist in the niche, including spindle-shaped N-cadherin⁺ osteoblasts (SNO), and oval-shaped osteoblasts. There is now abundant evidence that the osteoblasts, particularly SNOs, are a vital component of the HSC niche at the endosteal region and provide maintenance of a quiescent HSC phenotype^{62,63}. It was initially demonstrated that HSCs were located in close proximity to the osteoblasts supporting a possible functional relationship between the cells. The role of this endosteal region was determined using transplantation experiments that demonstrated engrafted HSCs became enriched within the endosteal region located in close proximity to the osteoblasts⁶²⁻⁶⁴. Further depletion experiments demonstrated that the

endosteal niche is important for homing and survival of HSCs and maintenance of long-term HSCs in a quiescent state^{65–67}. CXCL12-CXCR4 is one of the vital molecular interactions between osteoblasts and HSCs and facilitates both homing and maintenance of the HSCs. Inhibition of CXCL12 or CXCR4 caused HSC egression and mobilisation of HSCs from the niche^{68,69}. CXCR4 depletion also prevents HSC homing to the osteoblastic niche and increased mobilization into the circulation^{70–72}. Both BMP and PTHrP are key regulators of the endosteal niche in combination with other signalling pathways that mediate adhesion and quiescence, including angiopoietin-1/Tie2, thrombopoietin/MCL, N-cadherin/ β -catenin, vascular cell adhesion molecule 1 (VCAM1)/VLA4 and osteopontin^{64,65,73–75}.

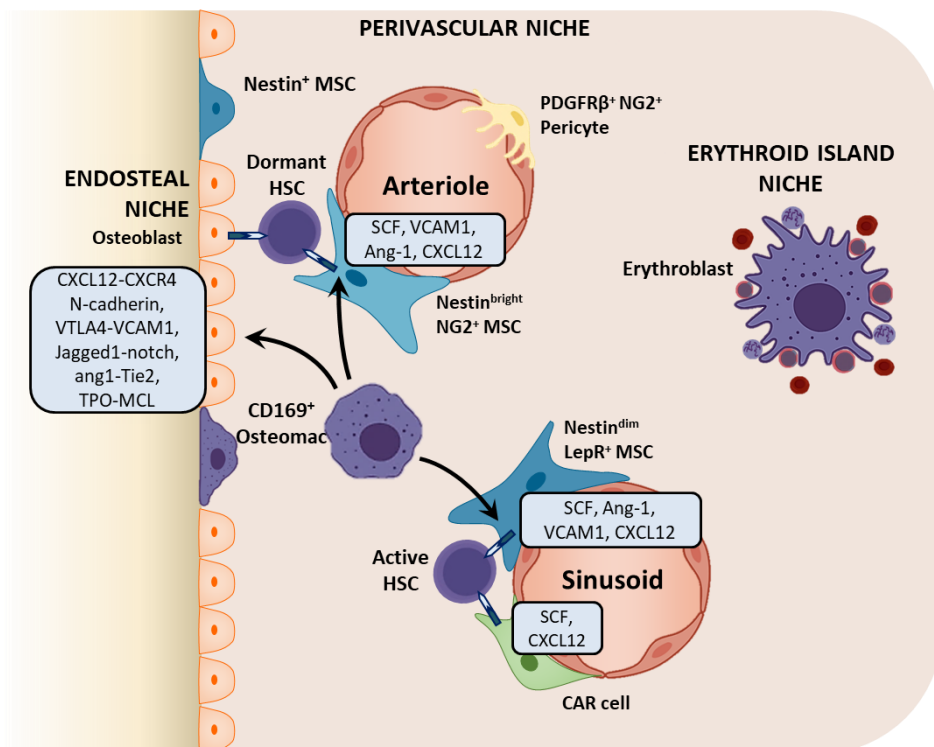


Figure 1.3.3: Components of the endosteal and perivascular HSC niche. The endosteal niche is composed of osteoblasts that form interactions with the HSCs to maintain a dormant, quiescent niche. Two separate perivascular niches exist around the arterioles and sinusoids with distinct nestin-expressing MSC populations and CAR cells. These maintain HSCs in a dormant and active phenotype, respectively. CD169⁺ Macrophages contribute to both of these HSC niches, as well as the erythroid island niche⁵⁸.

The perivascular niche is located around the arterioles, capillaries and sinusoidal vessels in the marrow, and consists of endothelial cells and several populations of mesenchymal cells, which promotes mobilisation, differentiation and proliferation of short-term HSCs. HSCs were first shown to be in direct contact with CXCL12-

producing cells, defined as CAR cells that were enclosed by endothelial cells or located near the endosteum⁷⁶. Whilst CXCR4-depletion or CAR cell-depletion using iDTR-mediated mechanism resulted in a reduced number of HSCs, it was unclear whether CXCL12-CXCR4 maintained HSCs proliferation⁷⁷ or a quiescent HSC pool⁷⁶. These CAR cells encompassed all cells expressing CXCL12 within the bone marrow, which may have differential effects on the HSCs. Several other CXCL12-expressing cell populations within the perivascular niche have since been described including Nestin⁺ MSCs, NG2⁺ pericytes and lepr⁺ cells, which have each been implicated in the regulation of HSCs^{37,78}. Mendez-Ferrer *et al*⁷⁸ first identified that Nestin⁺ MSCs located at both the perivascular and endosteal niche contribute a necessary component of the HSC niche. These cells were self-renewing cells with a multipotent lineage that could differentiate into the mesenchymal lineages both *in vitro* and *in vivo* confirming their identification as MSCs. Intravital microscopy of fluorescently labelled HSCs demonstrated that they home to Nestin⁺ MSCs at the perivascular region within the bone marrow after transplantation, and iDTR-induced depletion of Nestin⁺ cells prevents this homing effect resulting in reduced HSCs^{78,79}. Nestin⁺ MSCs are also important in HSC maintenance and MSC proliferation regulates HSCs expansion within the niche. The depletion of nestin⁺ MSCs resulted in a reduction of HSCs within the haematopoietic niche. Moreover, the administration of PTH, which has previously been shown to increase the number of HSCs and improve HSC homing as a result of expansion of osteoblast expansion⁶⁴, also enhanced the size of the proliferative fraction of the Nestin⁺ MSCs. In contrast, G-CSF treatment, previously indicated to mobilize the HSCs into the circulation, reduce HSC numbers within the niche and prevent osteoblast differentiation⁸⁰, also attenuated the proliferation of the nestin⁺ MSCs. Together this demonstrates the hormonal mechanisms that regulate the HSC expansion and maintenance, including PTH, could also be due to expansion of the nestin⁺ MSC niche⁷⁸, rather than the osteoblasts previously identified by Calvi *et al*.⁶⁴. Furthermore, Kunisaki *et al*.³⁷ demonstrated that distinct populations of Nestin⁺ cells exist within the bone: nestin^{bright} (perivascular) cells are rare, quiescent cells that also express NG2 and are found along the arterioles that are preferentially located in proximity to the endosteum; Nestin^{dim} (reticular) cells are more common cells associated with sinusoids, upregulate cell cycle-associated genes and exhibit higher proliferation³⁷. It was also determined using lineage mapping that perisinusoidal cells expressing lepr partially overlap with the nestin^{dim} MSCs, and deletion of stem cell factor (SCF) from lepr⁺ MSCs depletes HSCs in the marrow therefore is required for

maintenance of the HSCs⁸¹. It is thought that the arteriole niche in proximity with the endosteal niche maintains dormant, quiescent HSCs in the long-term through interactions with the nestin^{bright} NG2⁺ MSCs, similar to the endosteal niche, whereas Nestin^{dim} lepr⁺ MSCs induce proliferation and expansion of active short-term HSCs at the sinusoidal niche^{37,78}.

Furthermore, Crisan *et al.*³⁵ identified a population of perivascular pericytes that express NG2, PDGFR β , α SMA and CD146, and exhibit the criteria for defining MSCs. This suggests that classical pericytes may be the similar cell to perivascular MSCs and possibly a subpopulation of MSCs specific to this location³⁵. This is supported by the previous observation of NG2 expression within the perivascular Nestin^{bright} cells associated with arterioles but not the reticular Nestin^{dim} cells³⁷. It is therefore possible that NG2-expressing pericytes are in fact a specific subpopulation of Nestin⁺ MSCs express, which are involved in the perivascular HSC niche.

While advances in imaging and genetically modified mouse models to label these candidate cells has allowed the discovery of their roles in HSC maintenance, one of the main challenges is the identification of the cell types due to the likely considerable overlap between these populations. Although the exact identify of MSC is controversial, it is agreed that the MSC-like cells, whether they are defined as MSCs, CAR cells or pericytes, contribute to the HSC niche. As well as these cells being located near the HSCs, they also express factors involved in HSC quiescence including CXCL12 and SCF.

Osteomacs also localize to the HSC niche and have recently been identified as essential regulators of haematopoiesis. A population of CD169⁺ bone resident osteomacs act on the nestin-expressing niche through an unidentified cytokine to induce CXCL12 that in turn promotes HSC retention^{58,82}. Similarly, osteomacs also mediate the endosteal niche, and is disrupted in macrophage depleted mice observed as a reduction of osteoblast lineage cells and downregulation of HSC retaining factors, which subsequently induced HSC mobilization⁸³. It should also be noted that macrophages also contribute to the erythroid island niche, where they secrete factors such as erythropoietin to regulate erythropoiesis, however this niche has not been implicated in bone metastasis⁸⁴.

The final component of the niche is the sympathetic nerves, which directly interact with nestin-expressing cells in the marrow and express elevated levels of HSC maintenance

genes^{36,58}. Aberrant nerve conduction through the use of transgenic mice and pharmacological inhibitors results in dysregulated osteoblast function and reduced CXCL12 that coincides with HSC mobilization demonstrating a role at the endosteal niche⁸⁵.

1.3.3.2 Tumour cell seeding to the HSC niche

During the development of bone metastasis, the HSC niches are exploited by disseminated tumour cells (DTCs) and outcompete the HSCs for occupancy. Firstly, the cells within the niche, including bone marrow-derived MSCs and osteoblasts recruit DTCs to the bone via the release of chemoattractants. The DTCs subsequently seed and colonise the niche by the establishment of adhesion interaction, and are exposed to survival and chemoprotective factors to promote their persistence in the bone (figure 1.3.4).

The first direct evidence for bone metastatic cells utilising the HSC niche to colonise the bone marrow was provided by Shiozawa *et al.*⁵⁹ who demonstrated that prostate cancer cells localised to the endosteal niche and competed with the HSCs for the endosteal/osteoblastic niche. The presence of DTCs within the bone from subcutaneous prostate cancers prevented HSC engraftment to the niche after bone marrow transplantation suggesting that the DTCs from the primary tumour were occupying the niche. This is further supported by delayed engraftment after simultaneous transplantation of bone marrow cells and prostate cancers cells. Conversely, mobilisation of the HSCs from the niche using CXCR4 inhibitors to 'clear' the niche prior to injection of prostate cancer cells resulted in increased homing of prostate cancers niche. Of therapeutic relevance, the use of mobilising agents also induced mobilisation of prostate cancers from the niche back into the circulation where they were sensitive to treatment. Together, this demonstrates that prostate cancer cells occupy the endosteal niche either by mobilising the HSCs from the niche or increasing the size or number of endosteal niche⁵⁹ and is further supported by the specific homing and occupancy of the osteoblast-rich endosteal niche during bone colonisation in breast cancer models^{86–89}. Colonisation to the endosteal niche may be dependent on specifically senescent osteoblasts rather than the total population. A conditional mouse model that induced senescence in the osteoblasts was generated by inducing the expression of cell-cycle inhibitor p27^{kip1} was induced under the osteoblast-specific estrogen-responsive Cre recombinase. Breast cancer cells injected intracardiacally into these mice localized to the senescent osteoblasts and resulted in increased tumour

burden compared to littermate controls. This burden was correlated to the number of senescent osteoblasts⁹⁰.

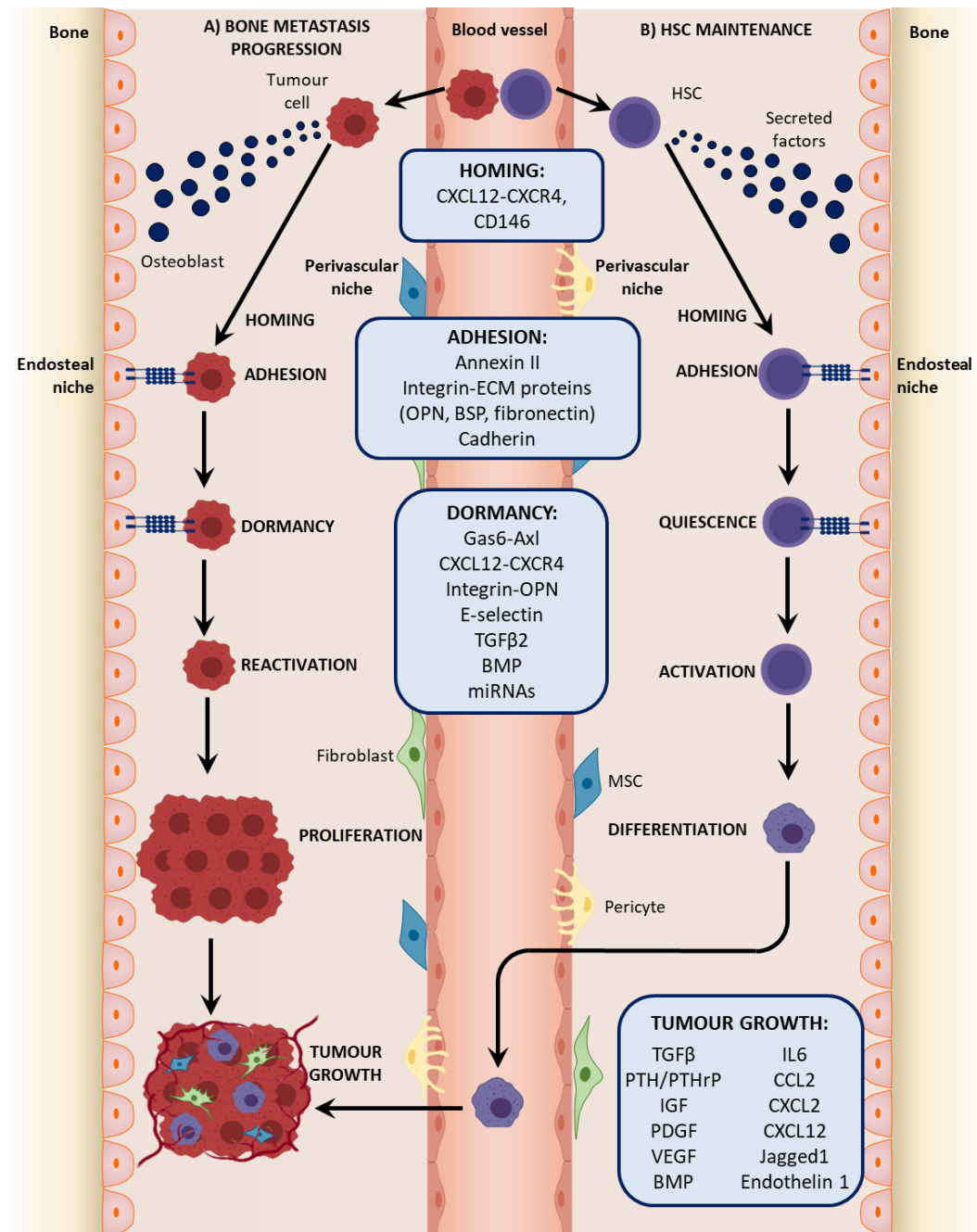


Figure 1.3.4: Involvement of the HSC niche in A) bone metastasis and B) HSC maintenance. DTCs and HSCs home to the endosteal and perivascular niche where they adhere to the osteoblasts and MSCs, respectively. Here they can remain dormant or quiescent, but are subsequently reactivated. DTCs proliferate to give rise to a metastatic lesion, whilst HSCs differentiate to give rise to the immune cells that enter the circulation⁵⁴.

Similarly, it was recently demonstrated melanoma cells home to the perivascular niche⁹¹. Moreover, PTH, which is expressed by bone metastatic cells, also enhances MSC proliferation and HSC expansion therefore may enlarge the perivascular to facilitate increased seeding capacity⁷⁸. The HSC niche is also mediated by tumour-derived factors, including BMP6, IL6, VEGF, and PTH, secreted from both the primary tumour and circulatory tumour cells (CTCs) that act on the stromal cells to expand the niche. These factors can either act directly upon the osteoblasts to induce an activated phenotype or secretion of chemoattractants, or can enhance MSC to osteoblast differentiation to expand the niche^{54,92}.

One of the key molecular pathways mediating tumour cell homing to the bone is the CXCL12-CXCR4 signalling axis, and has been extensively studied in breast and prostate bone metastasis as well as leukaemia^{59,93,94}. CXCL12 is released from both the osteoblasts and MSCs and the endosteal and perivascular niches, respectively, as described above, which acts as a chemoattractant to induce migration of the CXCR4⁺ DTCs to the bone. Silencing of CXCR4 in the HSC niche resulted in reduced extravasation and thus attenuated metastasis⁵⁹. Furthermore, subclones of breast cancer cells are selected from a heterogeneous population in the primary tumour by cancer-associated fibroblasts (CAFs) expressing CXCL12, and are therefore primed to survive in the CXCL12-rich microenvironment of the bone marrow⁹⁵. Of clinical relevance, expression of osteoblast-derived CXCL12 is enhanced by both radiation and chemotherapeutic agents such as cyclophosphamide and 5-fluorouracil, and therefore may enhance homing of tumour cells and thus bone metastasis⁷⁰.

A critical step during colonisation is the establishment of direct cell-cell interactions between the tumour cells and stromal cells to maintain contact at the niche. These interactions are similar to those used by the HSCs⁹⁶. Annexin-II is a phospholipid-binding membrane protein expressed by both osteoblasts and endothelial cells, and mediates HSC adhesion within the niche⁹⁷. Similarly, prostate cancer cells express the annexin-II receptor and utilise annexin-II to migrate and adhere to osteoblasts at the endosteal niche during colonisation. Inhibition of both annexin-II and annexin-II receptors reduced adhesion to osteoblasts and homing of prostate cancer cells to the endosteal niche *in vivo* and is associated with reduced metastasis⁹⁸.

Cadherins are important for tumour cell adhesion and colonisation of bone metastasis at the endosteal niche and is most strongly evidenced using an experimental model of

breast cancer. After intra-iliac injection to establish breast cancer bone metastasis, tumour adherence to the endosteal niche was mediated by heterotypic junctions formed between N-cadherin on the osteoblasts and E-cadherin on the tumour cells, which is associated with poor prognosis. The interaction between the cadherins resulted in activation of the mTOR pathway, which induces the outgrowth of single cells to micrometastases. Disruption of this interaction attenuated these effects resulting in attenuated bone metastatic burden^{88,99}. OB-cadherin is also elevated in human bone metastasis samples compared to normal and primary tumours, and is not expressed in metastasis of other organs. It is also elevated in bone metastatic breast and prostate cancer cells and facilitated the adhesion to osteoblasts *in vitro*¹⁰⁰. Downregulation of OB-cadherin in metastatic prostate cancer cells reduced their binding and migratory ability¹⁰¹ and attenuated bone metastasis burden *in vivo*¹⁰². Conversely, overexpression of OB-cadherin enhanced bone metastasis due to increase in early colonisation, which appears to be a bone specific effect as there was no effect on lung metastasis¹⁰⁰.

Similarly, gap junctions composed of connexin43 also form between the osteogenic niche and breast cancer cells during colonisation of the bone. This not only provides survival signals, but also permits the transfer of calcium from the osteoblasts to increase the intracellular concentration in the tumour cells, which subsequently promotes the persistence of the bone metastatic cells. Inhibition of these mechanisms prevented colonisation to the bone. Moreover, short-term, transient blockade during the seeding period significantly delayed the development of bone metastasis demonstrating an effective therapeutic strategy. Connexin43 is also elevated in bone metastasis samples and correlates with calcium signalling, which provides indication of translation to the clinic¹⁰³.

Within the sinusoidal niche, E-selectin is involved in homing of the breast cancer cells to the bone marrow. Blocking of E-selectin prevented adhesion to the vessel and migration into the bone marrow¹⁰⁴. Other molecules implicated in adhesion of tumour cells to the niche include integrins that facilitate binding to the extracellular matrix (ECM) proteins such as osteopontin and fibronectin⁵⁴.

The establishment of the interactions with the niche permits the bone metastatic cells to survive. This is often in a dormant state, and has been observed in both the perivascular and endosteal niche where non-proliferating cells resided adjacent to the niche^{105–107}.

In a model of myeloma, the use of intravital microscopy of the bone has demonstrated that label-retaining and therefore non-dividing tumour cells preferentially homed to the osteoblasts at the endosteal niche. This dormant phenotype in the cells co-localising with the endosteal niche was also confirmed by reduced Ki67 staining showing less proliferation, and gene set enrichment analysis that demonstrated the label-retaining cells exhibited downregulation of genes involved in cell cycle and replication whilst those involved in quiescence were upregulated. The interaction of the myeloma cells with the osteoblasts actively suppressed myeloma cell growth as also demonstrated *in vitro* where the co-culture of myeloma cells with osteoblasts or in osteoblast-derived conditioned media resulted in an increase of label-retaining cells compared to myeloma cells cultured alone. Moreover, the isolation of the label-retaining dormant myeloma cells from the bone into culture resulted in a diminished labelling and thus re-establishment of cell division, which was reversed when re-injected *in vivo*. Together, this demonstrates that the suppression of myeloma growth by the osteoblasts within the endosteal niche is reversible and can become activated and begin proliferation giving rise to an osteolytic lesion¹⁰⁷. A number of molecular interactions, including growth arrest-specific 6 (GAS6)-AXL, have been identified to mediate dormancy and render the bone metastatic cells resistance to chemotherapy as reviewed here by Graham and Qian⁵⁴. Whilst the mechanisms that govern the reactivation of dormant cells are unclear, DTCs within the niche often give rise to micrometastasis that subsequently develops into clinically overt metastatic lesions.

1.4 The tumour microenvironment

Tumours not only consist of the cancer cells themselves, but also other non-tumoural components, including immune cells, mesenchymal stromal cells, blood vessels, secreted molecules that are embedded in an ECM. Together, this makes up a tumour microenvironment (TME) in which the cancer cells exist (figure 1.4.1). It is now evident that complex communications between the non-tumoural host components and the tumour cells are established to create this unique environment that is vital in the development and progression of cancer¹⁰⁸. The role of the TME is of great interest therapeutically as disruption of the interactions with the tumour cells attenuates tumour progression, and has attracted particular attention as the stromal cells are more genetically stable relative to the tumour cells and thus making them an effective therapeutic target.

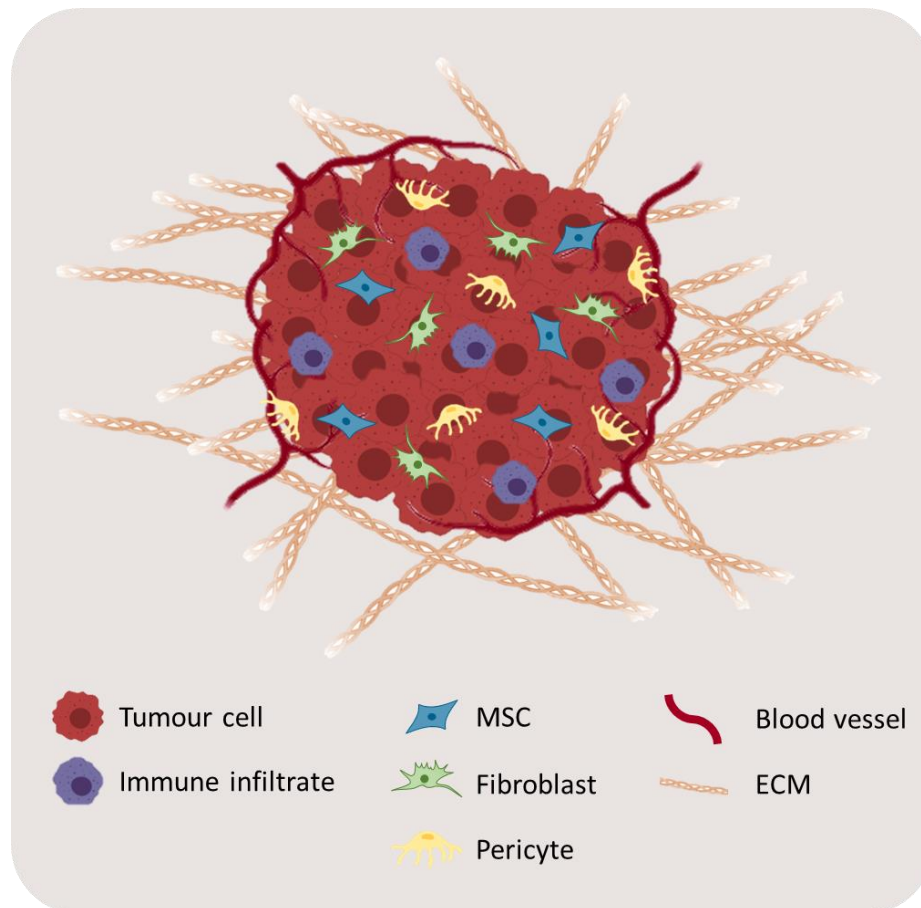


Figure 1.4.1: Components of the tumour microenvironment. Cancer cells are surrounded by a complex microenvironment consisting two cellular compartments: the mesenchymal compartment contains MSCs, pericytes, fibroblasts and endothelial cells; the immune compartment contains myeloid cells (macrophages, MDSCs, neutrophils) and lymphoid cells (T cells, B cells and NK cells). These cells are embedded within an ECM and exposed to secreted factors¹⁰⁹.

Tumours have therefore been described as ‘wounds that do not heal’ sharing many features with the inflammatory response. This includes the recruitment of cells predominantly immune and mesenchymal populations from the bone marrow, which infiltrate the tumour and exert pro-tumorigenic effects. The cells within the TME collaborate together and not only enhance the growth of primary tumours, but also facilitate metastasis dissemination to secondary sites with each stage of the metastatic cascade being promoted (figure 1.4.2).

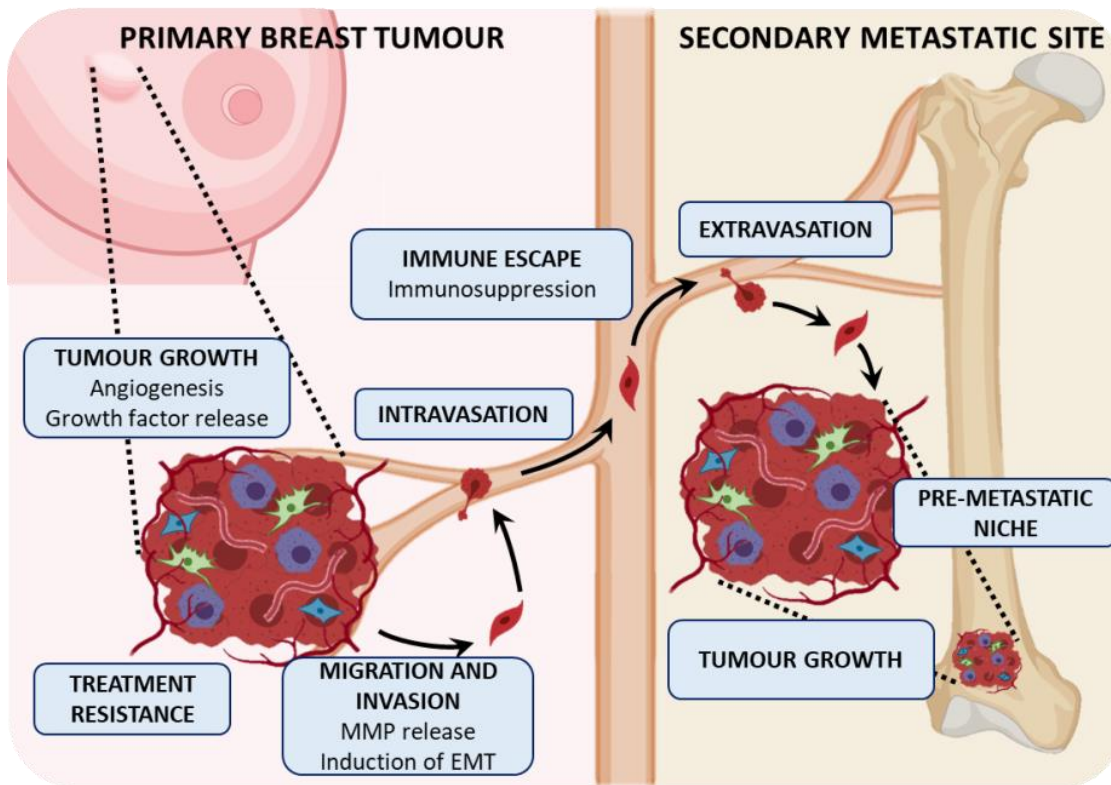


Figure 1.4.2: Promotion of tumour progression by the TME throughout the metastatic cascade. Within the primary tumour, components of the TME secrete factors that promote growth by either acting directly on the tumour cells to enhance proliferation, or by inducing angiogenesis that permits further delivery of nutrients and oxygen to the tumour. Protective factors are also released that cause resistance to treatments, including chemotherapy and immunotherapy. An immunosuppressive environment is also created within the tumour by repression of an anti-tumour immune response, and is mediated by inhibitory factors from both the tumour cells and other components of the TME. The TME also induces a migratory phenotype in the tumour cells via the EMT, as well as the production of MMPs that together induces migration and invasion from the primary tumour. Intravasation is also promoted by the formation of cellular structures around the blood vessels consisting of macrophages and endothelial that act as a gateway into the circulation. At the metastatic site, the TME forms a pre-metastatic niche that both attracts the tumour cells to the secondary organ and forms adhesions that mediates seeding and colonisation. This niche also provides survival factors to promote persistence of the tumour cells at the secondary site. The formation of the niche is mediated by both the tumour cells as well as other components of the TME, and can also be expanded by the effects of cancer treatments. Subsequently, components of the TME promote tumour growth within the secondary site, giving rise to a metastatic lesion. The effect of the TME is distinct between the primary tumour and secondary metastasis.

1.4.1 Components of the TME

The cellular components of TME can be compartmentalised into the mesenchymal compartment and the immune compartment consisting of a variety of myeloid and

lymphoid populations. These cells are embedded in a rich ECM composed of proteins such as collagens and fibronectin, which is diverse between tumours and highly dynamic. The ECM not only acts as a scaffold for cell migration and invasion, but also activates cellular signalling pathways through interactions with ECM receptors on the cell surface, which leads to alterations in cell survival and behaviour¹¹⁰. The cells within the TME investigated throughout this project are described below.

1.4.1.1 Mesenchymal compartment

The mesenchymal compartment of the TME is composed of MSCs, pericytes, fibroblasts and endothelial cells as well as osteoblasts in the bone. These cell populations are recruited to or activated by the tumour where they exert tumour-promoting effects via molecular interactions summarised in figure 1.4.3. As described previously, MSCs are a poorly defined population of cells and overlap with other MSC-like cells, such as pericytes, which complicates the interpretation of their role in cancer. Nevertheless, it has been demonstrated that bone marrow-derived MSCs are recruited to tumours via the secretion of chemoattractants where they persist and were previously utilised as drug delivery vectors due to this property^{111–113}. Within the tumour, MSCs exhibit divergent roles displaying both anti- and pro-tumorigenic effects in different cancer models. However, the number of studies demonstrating an anti-tumour role is low and restricted to limited cancer types^{114,115}. In contrast, there is abundant evidence demonstrating a tumour-promoting role of MSCs throughout the metastatic cascade¹¹⁶. For example, Karnoub *et al.*¹¹⁷ demonstrated the co-injection of weakly metastatic breast cancer cells with bone marrow-derived MSCs increased the dissemination to the lung via the secretion of MSC-derived CCL5 that acts upon CCR5 on the breast cancer cells¹¹⁷. MSCs can also induce an immunosuppressive TME through the secretion of soluble factors such as IL6 and exosomes that act upon immune cells including macrophages and T cells^{118,119}. MSCs have also been implicated in the induction of chemotherapy resistance in ovarian cancer and breast cancer via the release of MSC-derived fatty acids and IL8, respectively^{120,121}. Similarly, cells defined as PDGFR β ⁺ pericytes also promote tumour growth and metastasis¹²².

Fibroblasts are another significant mesenchymal cell within the TME and are associated with poor prognosis in cancer patients¹²³. Within normal tissues, fibroblasts are inactive cells, however, during inflammatory responses such as wound healing and fibrosis, they are activated becoming myofibroblasts. Fibroblasts within the TME, defined as cancer-associated fibroblasts (CAFs), share an activated phenotype similar

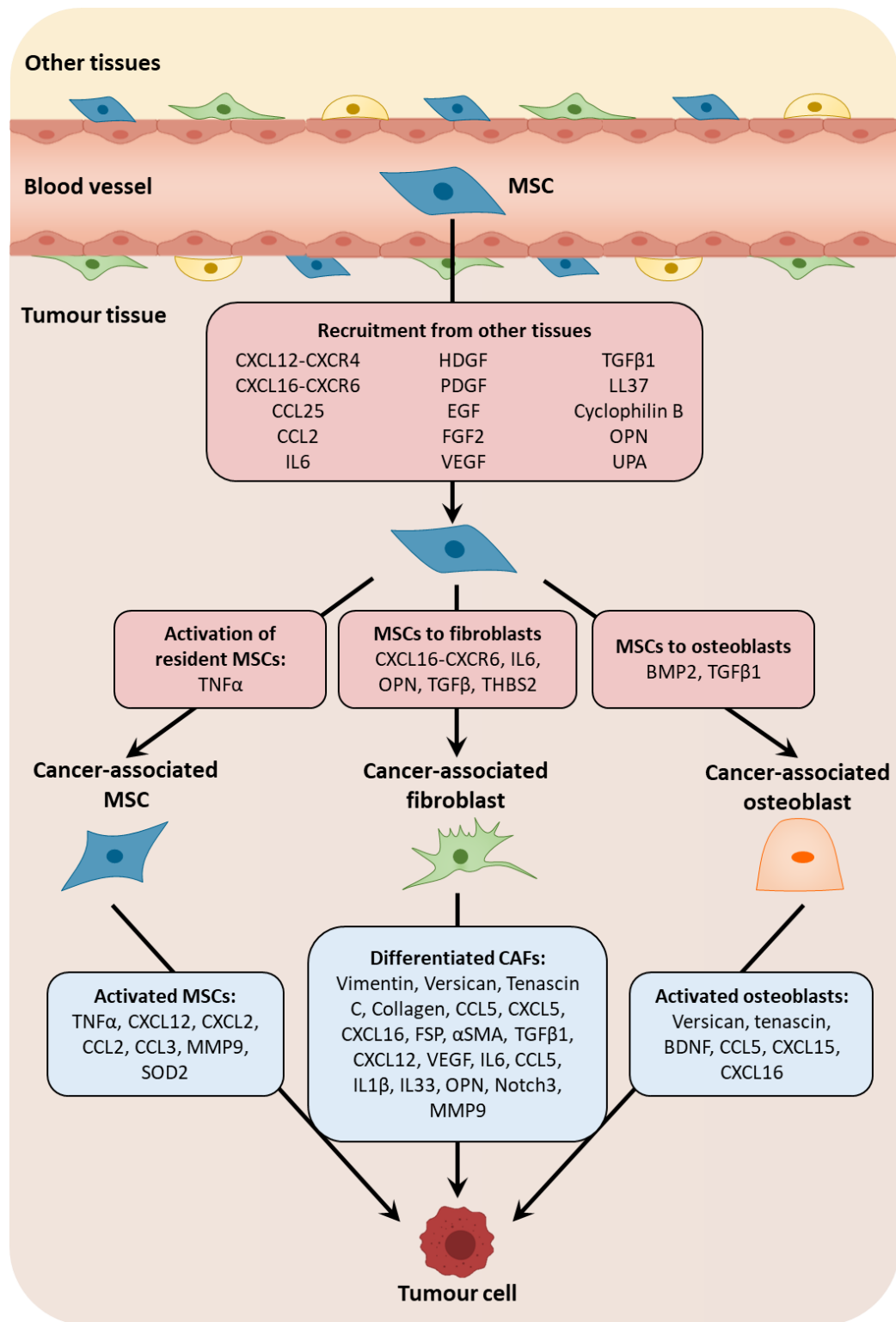


Figure 1.4.3: Role of MSCs in the TME. MSCs are recruited to the TME from other tissues such as the bone, and can be activated or differentiate into fibroblasts or osteoblasts. This process is dependent on tumour-derived factors that act on the MSCs. Subsequently the MSCs, fibroblasts or osteoblasts secrete tumour-promoting factors that mediate tumour progression and metastasis via the promotion of growth, invasion and metastatic niche⁵⁴.

to myofibroblasts¹²⁴. However, whilst deactivation and removal of myofibroblasts is coordinated with the resolution of the wound, CAFs remain in a permanently activated state, and continue to secrete factors involved in tumorigenesis including ECM factors and cytokines^{123,125,126}. Moreover, CAFs isolated from prostate cancer display enhanced tumour-promoting properties compared to fibroblasts isolated from their normal counterpart tissues, suggesting CAFs are also more pro-tumorigenic than normal fibroblasts¹²⁶. During activation, myofibroblasts acquire expression of alpha-smooth muscle actin (α SMA), fibroblast activated protein (FAP), fibroblast specific protein (FSP) and PDGFR α , which are utilised as markers to identify CAFs, although are not specific and overlap with other cell types¹²⁷. It was also recently determined that CAFs can also arise from tumour-induced differentiation of MSCs and pericytes and represent 20-40% of the total CAF population depending on tumour type^{122,127–129}. CAFs contribute to the maintenance of the ECM through deposition of new ECM components such as collagens, laminins and fibronectin, as well as secretion of MMPs to remodel the existing ECM. Furthermore, they also secrete cytokines such as CXCL12, VEGF, hepatocyte growth factor (HGF) and TNF α that recruit tumour-promoting immune cells, induce an immunosuppressive environment, and enhance angiogenesis¹²⁷. Together, this exerts a tumour-promoting effect that significantly enhances to tumour progression.

1.4.1.2 Immune compartment

The immune system was traditionally thought to mediate an anti-tumour response in order to eradicate cancer. However, over the last few decades it has become apparent that as cancer evolves it engages with and manipulates the immune cells. This can result in the generation of an immunosuppressive environment that permits tumour growth, or can induce immune cell phenotype that supports cancer survival and development via the secretion of tumour-promoting cytokines and growth factors.

1.4.1.2.1 Myeloid cells

Intratumoral macrophages, defined as tumour-associated macrophages (TAMs), represent a dominant population in the TME. Macrophages are differentiated cells of the myeloid lineage forming part of the innate immune system. Whilst they were initially identified by their ability to undergo phagocytosis to eliminate cell debris and infectious agents, macrophages reside in all tissues where they are also vital in development, homeostasis and tissue regeneration and repair, and have been implicated in almost all

pathological conditions¹³⁰. Resident macrophages are derived from the embryonic yolk sac and subsequently the fetal liver, defined as primitive haematopoiesis, and undergo self-renewal throughout adult life to maintain the population. Conversely, macrophages recruited to tissues are derived from HSC-derived monocytes within the bone marrow. During development, definitive HSCs emerge from the aorta-gonad-mesonephros, and migrate to the fetal liver to give rise to all immune lineages during embryonic development. After gestation, the bone marrow serves as the major haematopoietic origin giving rise to monocytes that are recruited to the tissue usually in a CCL2-CCR2 dependent manner, where they subsequently polarise into macrophages¹³¹. Terminally differentiated macrophages are identified by the expression of CD11b, F4/80 and colony-stimulating factor 1 receptor (CSF1R) in mice, and CD68, CD163 and CD115 in humans¹³².

The majority of TAMs are derived from bone marrow-derived monocytes that are recruited to the tumour via the secretion of chemoattractants, predominantly CCL2, where they undergo differentiation into a TAM dependent on the cues from the TME. Resident TAMs are also able to proliferate and become activated to give rise to TAMs¹³⁰. TAMs are adaptable cells responding to cues within the TME, particularly from the tumour cells, to induce distinct TAM populations that can either impair or promote tumour progression, depending on their functional state. Macrophages have previously been classified on the basis of their involvement in the immunological response. 'Classically activated' M1 macrophages are involved in type 1 helper T (Th1) cell response to pathogens, and are activated by interferon gamma (IFN γ) and engagement of Toll-like receptors (TLRs). They are characterised by the expression of major histocompatibility complex (MHC) II and secretion of proinflammatory cytokines including IL2 and TNF α . Conversely, 'alternatively activated' M2 macrophages are involved in Th2 T cell response cell responses such as wound healing and are activated by IL4 and IL13^{133–135}. It was previously thought that M1-like macrophages exhibited a tumour-inhibitory phenotype, whereas tumour-promoting TAMs were skewed towards an M2-like phenotype¹³⁶. However, gene profiling experiments of TAMs isolated from cancers demonstrated that tumour-promoting TAMs are instead composed of several distinct populations that each exhibit specific functions in promoting cancer progression and metastasis (figure 1.4.4), reviewed by Qian and Pollard¹³⁴. Moreover, TAMs exhibit diversity between tumour types as well plasticity between metastatic sites. TAMs isolated from primary tumour are not only different to

macrophages from the metastatic site, defined as metastasis-associated macrophages (MAMs), but also between different metastatic sites^{130,137}.

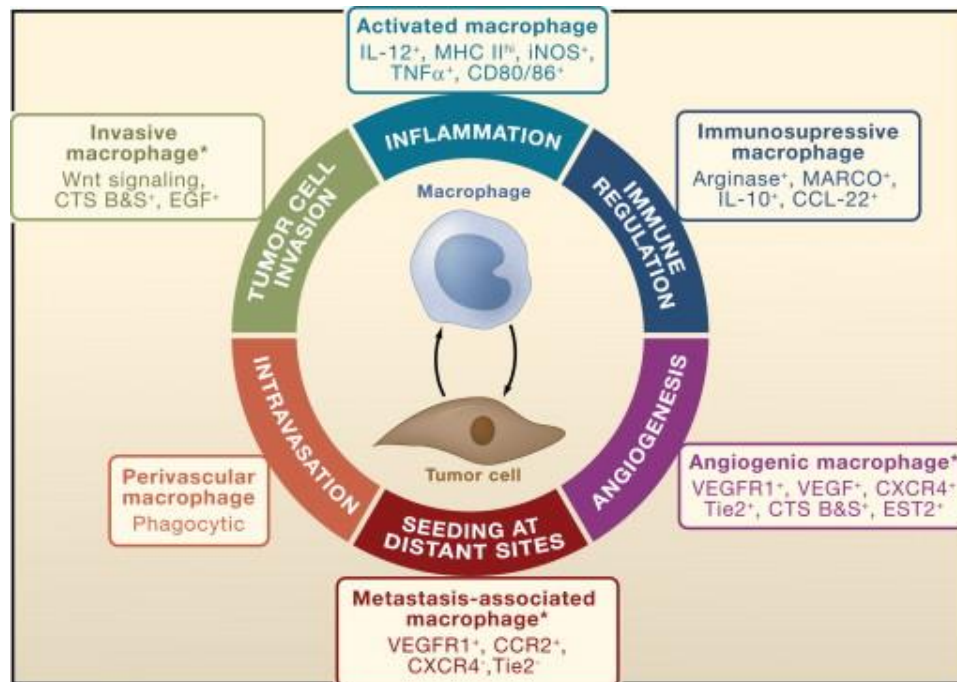


Figure 1.4.4: Pro-tumoural functions of TAMs in the TME. Subtypes of TAMs exist within the TME dependent on environmental stimuli. The resulting phenotypes exert specific effects that enhance different aspects of tumour progression and metastasis¹³⁴.

TAMs exert pro-tumorigenic effects in almost all cancers and are associated with a poor prognosis, particularly in breast, prostate, ovarian and cervical cancers¹⁰⁸. The role of TAMs has been confirmed in many cancers where macrophage depletion in tumour-bearing mice using either *Csf1^{op/op}* mice deficient in colony stimulating factor 1 (CSF1) or pharmacological depletion with liposome clodronate results in significantly less infiltration of TAMs. This was associated with attenuated primary tumour growth and metastasis due to reduced tumour angiogenesis, tumour cell invasion, matrix remodelling and immune suppression^{138–140}. Protumour TAMs are also immunosuppressive through the secretion of soluble factors as well as expression of immune checkpoint ligands that suppress CD8⁺ T cells, CD4⁺ Th1 cell cells and natural killer (NK) cells, whilst activating T regulatory cells (Tregs) and Th2 cells¹³⁶. TAMs also mediate resistance to chemotherapy^{136,141}. Analysis of tumours from patients undergoing neoadjuvant therapy shows that chemotherapy induces a infiltration of

TAMs as the tumour exhibit a higher abundance of TAMs compared to those who only receive surgery¹⁴². Administration of CSF1 inhibitors can restore chemotherapy sensitivity in murine models of breast cancer resulting in reduced tumour burden¹⁴³.

MAMs are recruited to the metastatic site where they promote the development of metastasis. For example, in a murine model of breast cancer, monocytes were recruited to the lung in a CCL2-CCR2 dependent manner where they promote metastatic seeding and growth, which was inhibited in tumours established in macrophage depleted mice. Further studies demonstrated that the seeding was via the secretion of VEGF^{144,145}.

Targeting TAMs therefore seems an attractive anti-cancer therapy. Numerous inhibitors of the CSF1-CSFR1 pathway have been trialled in the clinic and showed success in improving the response to chemotherapy and suppressing metastasis. However, as these non-specifically kill all macrophages, patients these exhibited significant adverse effects. Novel strategies have therefore been considered including targeting of TAM-specific molecules as well as reprogramming the TAM phenotype towards and M1-like, tumour-inhibitory phenotype via inhibition of CD40, TLR7 and TLR9^{130,136}.

Myeloid-derived suppressor cells (MDSCs) are a relatively newly discovered heterogeneous population of myeloid cells also present in the TME and have emerged as important regulators of immunosuppression in cancer. They are defined by the expression of CD11b⁺ and Gr1⁺ in mice and are further divided into two main subsets based on their morphology, density and cell surface markers: polymorphonuclear (PMN)-MDSCs are neutrophil-like cells defined as CD11b⁺ Ly6G⁺ Ly6C^{low} in mice and CD15⁺ CD14⁻, CD33^{dim}, HLA-DR⁻ in humans whereas monocytic MDSCs are monocyte-like cells defined by CD11b⁺ Ly6G⁻ Ly6C^{high} and CD15⁻ CD14⁺, CD33⁺, HLA-DR⁻^{146,147}. Both function as immunosuppressive cells that inhibit the adaptive immune response the suppressing CD4⁺ and CD8⁺ T cells and are accumulated in cancer patients and murine models^{108,148,149}. Whilst their role in cancer progression is less understood, they have been implicated in generation of an immunosuppressive environment. S100A9 upregulated in colorectal cancer induces recruitment and expansion of MDSCs in the tumour, whereas mice lacking S100A9 exhibited an immune response against the tumour, which was reversed by administration of MDSCs isolated from tumours in wildtype mice¹⁵⁰. MDSCs also home to the invasive front of breast cancer mediated through the CXCL12-CXCR4 and CXCL5-CXCR2 pathways

where they facilitate tumour cell invasion and metastasis to the lung via the elevation of MMPs. Treatment of tumour-bearing mice with CXCR2 or CXCR4 antagonists reduced the lung metastasis *in vivo*¹⁵¹.

1.4.1.2.2 Lymphoid cells

T cells are the second most abundant immune cell type in the TME and their roles have been extensively studied. They are components of the adaptive immune system and encompass several subpopulations that function as either mediators or effector of a response depending on the context and microenvironmental cues. T cells consist of CD4⁺ or CD8⁺ T cells populations, known as helper and cytotoxic T cells, respectively. CD8⁺ T cells are responsible for the destruction of intracellular virus infections, as well as tumour cells, and recognise MHC I molecules expressed on the surface of all nucleated cells¹⁵². CD4⁺ T cells, specifically T helper cells, recognise antigens bound to MHC II molecules expressed by antigen-presenting cells including macrophages and dendritic cells, and release cytokines that mediate the response of other immune cells. This includes maturation of B cells and activation of T cells and macrophages. This response is dependent on the context: Th1 cells secrete proinflammatory cytokines such as IFN γ and initiate a response against intracellular infections and cancer by activation of the cytotoxic CD8⁺ T cells; Th2 cells secrete IL4 that mediates B cell response; and Th17 cells that secrete IL17. Moreover, a subpopulation of CD4⁺ T cells exist as Tregs and are identified by the expression of CD25 and Forkhead box O3 (FOXOP3)^{+/}. These cells function as an immunosuppressive cell to restrict the T cell response during the final stages of a reaction and prevent an autoimmune response¹⁵³.

This contextual response also extends to intratumoural T cells where CD4⁺ Th1 T cells aid CD8⁺ T cells in tumour rejection; CD4⁺ Th2 T cells prevent CD8⁺ T cell activation to promote tumour development¹⁵⁴. After activation by antigen-presenting cells, CD8⁺ T cells orchestrate an anti-tumour immune response and target cancer cells for destruction via the secretion of perforin- and granzyme-containing granules^{155,156}. This effect forms the basis of chimeric antigen receptor (CAR) T cell therapy where patient T cells are engineered to recognise the cancer cell more effectively. CD4⁺ Th1 cells also mediate an anti-tumoural response through the secretion of proinflammatory cytokines IL2, TNF α and IFN γ . These not only activate cytotoxic CD8⁺ T cells but also enhance the anti-tumour activity of macrophages and NK cells¹⁵³. The presence of tumour-infiltrating CD8⁺ T cells and Th1-cytokines is associated with favourable prognosis and increased overall survival in many cancers¹⁵⁷. However, an immunosuppression is

often observed in the TME to prevent destruction by the cytotoxic T cells, and is mediated by several aspects. Firstly, Tregs are abundant in tumours and suppress the anti-tumour response by impairing the activation and expansion of T cells through the secretion of immunosuppressive cytokines such as TGF β and IL10. Anti-tumour T cell responses are restored by Treg depletion¹⁵⁸. Other components of the TME, including TAMs, MDSCs and CAFs also contribute to immunosuppression¹⁵⁹. Additionally, immune checkpoint molecules including programmed cell death protein 1 (PD1), programmed death-ligand 1 (PDL1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4), which suppress T cell function, are upregulated in cancer and are associated with immune evasion, which will be discussed later¹⁵⁹.

Other lymphocytes, including NK cells belonging to the innate lymphoid immune response are also activated in cancer tissues and triggers cancer cell destruction. The association between NK cell activity and suppression of tumour development has been demonstrated in several mouse models¹⁶⁰. In cancer patients there is a correlation between NK cell abundance and a positive outcome, whereas reduced NK cell function is associated with worse prognosis. However, cancers often exhibit resistance NK cell-mediated killing due to downregulation of ligands that activate NK cell receptors¹⁶⁰. Whilst the cytotoxic lymphocytes, including CD8⁺ T cells and NK cells, are able to induce an anti-tumour immune response, the TME predominantly exhibits immunosuppression, including inhibition of the cytotoxic cells and activation of the regulatory cells. This therefore permits further tumour growth.

Together, the components of the TME described here predominantly exert a tumour-promoting effect. A more detailed description of the roles throughout the development of primary tumour and during the metastatic cascade is reviewed by Quail and Joyce¹⁰⁹, and will be discussed in further detail throughout the thesis.

1.5 Aims and hypothesis

The role of the TME has been extensively researched and it is now apparent that its components exert tumour-promoting effects. However, much of this previous research has been performed in primary cancers with limited research into the role at the metastatic site. The TME-mediated mechanisms that underlie metastatic disease, particularly in the bone, thus remain poorly understood.

The bone marrow niche is of a particular interest due to the analogous roles during both homeostatic processes and establishment of bone metastasis. Regulatory interactions between the macrophages and mesenchymal lineage within the endosteal and perivascular niche important for HSC maintenance and bone remodelling may play a similar role throughout the progression of bone metastasis. This includes early stage seeding and late stage growth, as well as providing a niche to facilitate treatment resistance. We will therefore investigate the contribution of the TME throughout the development of breast cancer bone metastasis, with a focus on the following aims and hypotheses:

- **The contribution of macrophages to the early stage seeding of breast cancer cells in the bone during colonisation.** As macrophages have previously been demonstrated to promote breast cancer seeding and colonisation to the lung^{144,145}, we will test the hypothesis that macrophages also contribute to early stage breast cancer cell seeding to the bone by performing bone metastasis assays in macrophage depleted mice. We will also develop novel techniques to image the bone, including an intravital imaging platform that will permit 3D imaging of tumour cell seeding in real time. This will determine whether tumour cells seed directly to the macrophages or if they modulate an alternative niche such as the previously identified endosteal niche.
- **The role of the mesenchymal lineage, including Nestin⁺ MSCs and SP7⁺ osteoblasts, during late stage breast cancer bone metastatic growth.** As MSCs have been previously implicated in tumour growth and are an important population present in the bone, we will determine the contribution of the mesenchymal lineage to late stage growth of breast cancer bone metastasis. To do this, we will establish bone metastasis in transgenic mice that allow the

conditional depletion of nestin⁺ MSCs or SP7⁺ osteoblasts during late stage growth.

- **The effect of chemotherapy on breast bone metastasis, with a particular focus on paclitaxel and doxorubicin, and the influence of the TME during treatment.** These chemotherapies are both first line treatments for breast cancer and exhibit low response rates with prevalent relapse in the bone. Moreover, they are often used as an integral component of palliative care but exhibit resistance and only partial responses. We will subsequently explore the role of macrophages, lymphocytes and mesenchymal cells in the response to chemotherapy. By identifying the cells responsible, it may offer insight into potential therapeutic strategies to overcome the disease.

To do this, we will utilise a novel model of breast cancer bone metastasis. A murine and human bone metastatic breast cancer cell line was previously derived from the mammary tumours induced by the mammary specific expression of the Polyoma Middle T oncoprotein from the MMTV promoter (MMTV-PyMT) and the MDA-MD-231 breast cancer cell line, respectively. Tumour cells were selected through rounds of injection and recovery to the bone to establish a subclone with increased metastatic potential to the bone. These cell lines represent a triple negative cell phenotype^{161,162}, which is the most aggressive subtype of breast cancer and has the most limited treatment options. Bone metastasis will be established *in vivo* via intracardiac and intrailiac injection of these cells, and will allow us to explore the role of the TME independent of the influence of a primary tumour. We will concentrate on the mesenchymal lineage and macrophage component of the TME, which we will define using the 'classical' markers illustrated in figure 1.5.1 for the purpose of this project, although it is important to highlight the overlap between the populations and will be discussed throughout.

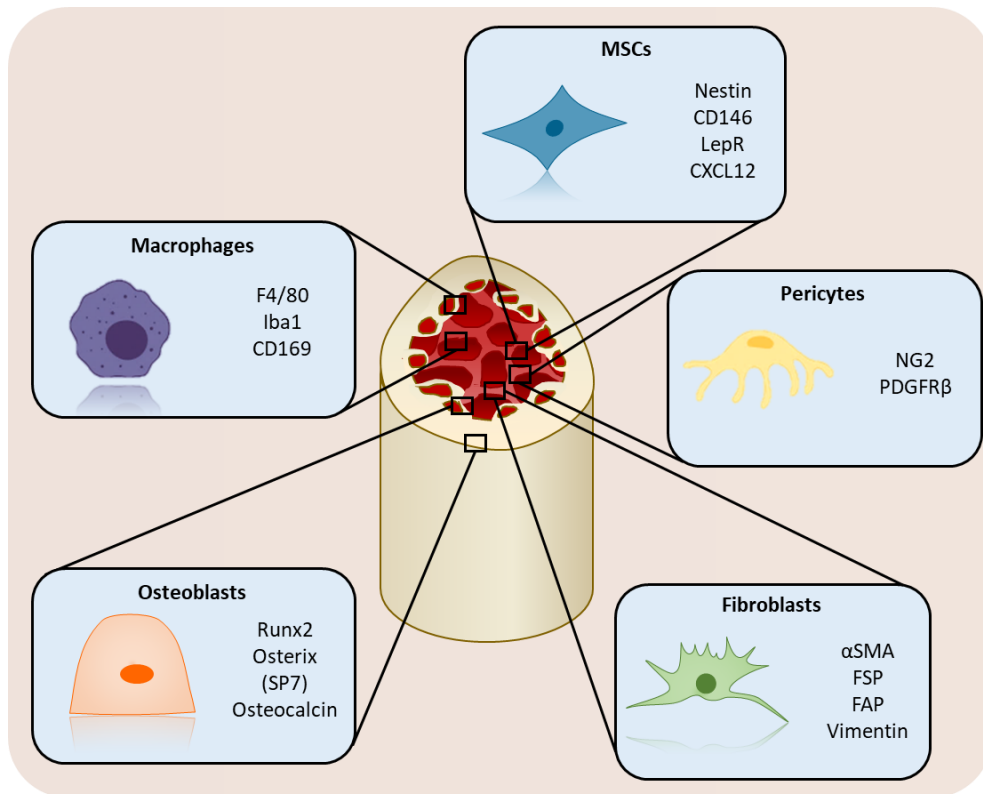


Figure 1.5.1: Cells of the TME in bone metastasis and classical markers used for identification.

Chapter 2 Materials and Methods

2.1 Cell culture

Sterile conditions were used during all cell culture. All cultures were maintained with 5% CO₂ in a culture incubator at 37°C. Cells were passaged using 0.25% EDTA-trypsin at appropriate confluence. The cultured cell lines (except primary cultures) were confirmed to be mycoplasma negative by PCR testing at regular intervals throughout the project.

Breast cancer cell lines were previously produced and maintained in our laboratory. MetBo2 cells are a bone metastatic subclone of Met1 mouse mammary tumour cells derived from the MMTV-PyMT tumour in FVB/N mice. 1833 cells are a bone metastatic subclone of the MDA-MB-231 cells. Both subclones were selected *in vivo* through rounds of intracardiac injection and recovery from the bone to establish a subclone with increased metastatic potential to the bone^{163–165}. Both were cultured in complete culture media consisting of Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). C3H10T12 cells, which are an embryonic MSC cell line, were cultured in α -minimum essential media (α MEM) (10% FBS, 1% P/S) supplemented with 1% glutamate and 1% sodium pyruvate¹⁶⁶.

2.2 MSC and fibroblast isolation and culture

Primary MSCs were isolated from murine bone marrow under sterile conditions as described previously¹⁶⁷. Briefly, bones were dissected and the proximal and distal ends removed using scissors. The bone marrow was flushed from the bone using a syringe and collected in complete α MEM media, supplemented with 10% FBS and 1% P/S. The cell suspension was centrifuged and resuspended in complete α MEM media in culture flasks. After 3 days, non-adherent cells were removed from culture by washing in sterile phosphate buffered saline (PBS) and replacing with new α MEM. Cells were passaged once confluence was reached. MSCs were selected using selective trypsinisation due to the difference in adherence properties of the MSCs.

Human primary MSCs were isolated from patient bone marrow samples, kindly received from Dr William Cawthorn, University of Edinburgh. Patient bone marrow samples were collected from patients undergoing surgery and informed consent was obtained from all donors. Bone marrow cells were cultured in DMEM media, supplemented with 10% FBS, and 1%P/S, at the concentration of 5×10^4 cells/cm². After 3 days, non-adherent cells were removed and medium replaced. The media was changed every 3-4 days until confluent. Cells were passaged when confluence was reached.

Human fibroblasts were kindly provided by Dr Thomas Hughes, University of Leeds. Normal fibroblasts (NF) were previously isolated from healthy breast tissue of two patients undergoing surgery (NF1 and NF2). Cancer-associated fibroblasts (CAFs) were isolated from the tumour region of patients undergoing breast cancer removal surgery¹⁶⁸. NFs and CAFs were cultured in DMEM supplemented with 10% FBS and 1%P/S.

2.2.1 Flow cytometry analysis of MSCs

The purity of the MSC culture was confirmed using flow cytometry analysis. Cells were trypsinised and resuspended at 250×10^3 - 500×10^3 cells/2ml in FACS buffer (1% bovine serum albumin (BSA) in PBS). Cells were centrifuged and the supernatant removed. The cells were incubated with the antibodies for 30minutes at 4°C in the dark (Sca1-APC BioLegend D7; CD29-PE eBioscience HMb1-1; CD29-APC BioLegend TS2/16; CD31-PE BioLegend WM59; CD105-PE eBioscience MJ7/18; CD105-PerCPCy5.5 BioLegend 43A3; CD45-PerCP/Cy5.5 BioLegend 30-F11; CD45-APC/Cy7 BioLegend 30-F11; CD44-APC/Cy7 BioLegend IM7; CD11b-AF488 BioLegend M1/70; CD34-BV605 BioLegend 581). Cells were washed and resuspended in FACS buffer. Compensation was set up using compensation beads, which were incubated with the same antibodies for 15 minutes at 4°C in dark. Flow analysis was performed on a 6L LSR Fortessa (BD) and analysed using FlowJo V10 (BD).

2.2.2 Differentiation of MSCs

Differentiation media in α MEM was used to differentiate MSCs into different lineages. Osteoblastic differentiation was induced by culturing the MSCs for 4 weeks in 5mM β -glycerophosphate, 50ug/ml ascorbic acid and 10nM dexamethasone with 10% FBS in α MEM with P/S. Adipocyte differentiation was induced with 500uM IBMX, 10ug/ml

insulin and 50nM dexamethasone with 10% FBS in α MEM with P/S¹⁶⁹. Fibroblast differentiation was induced by 10uM 5-azacitidine with 10% FBS α MEM¹⁷⁰.

Differentiation to osteoblasts, fibroblasts and adipocytes was measured using alizarin red and ALP staining, α SMA staining and Oil Red O staining, respectively.

2.3 Osteoblast isolation

Murine osteoblasts were isolated from calvaria of up to 4 day old mouse pups. Under sterile conditions, calvaria were dissected from the mice and washed in ~6ml Han's balanced salt solution (HBSS). The calvaria were digested in 2-3ml 1mg/ml collagenase for 10min at 37°C on a shaker, and the supernatant discarded. The digestion step was repeated, the supernatant retained as fraction 1. The calvaria was washed in 6ml PBS, which was added to fraction 1. The calvaria were shaken in 2-3ml of 4mM Ethylene Diamine Tetra Acetic Acid (EDTA) at 37°C for 10 minutes, the supernatant retained as fraction 2. The calvaria was washed in ~6ml HBSS, which was added to fraction 2. Fraction 1 and 2 were centrifuged at 2000rpm for 5 minutes and the supernatant removed. Both fractions were combined to make a single cell suspension and resuspended in culture flasks in α MEM supplemented with 10% FBS.

To remove OBs from within the bone, the digestion of calvaria in collagenase was repeated for 30 minutes at 37°C. The supernatant was removed and centrifuged at 2000rpm for 5 minutes and suspended, as fraction 3. This was added to the culture flasks containing fractions 1 and 2. After at least 3 hours, the media was replaced with fresh complete media.

2.4 Bone marrow macrophage isolation

Primary macrophages were isolated from murine bone marrow under sterile conditions. Bones were dissected, and the proximal and distal ends removed using scissors. The bone marrow was flushed from the bone using a syringe and collected in complete DMEM supplemented with 10% FBS, 1% L-glutamine and 1% P/S. The cell suspension was centrifuged and resuspended in complete DMEM with 20ng/ml M-CSF in an uncoated petri dish. After 24 hours, non-adherent macrophages were transferred to a new uncoated petri dish. Media was replaced every 3 days and macrophages were passaged when confluence was reached. Macrophages were ready for use after 7 days in culture.

2.5 Establishment of labelled MetBo2 cells

2.5.1 Qtracker™ Cell labelling

MetBo2 cells were labelled with Qtracker™ Cell labelling Kit (Q-dots) using the protocol provided. The labelling solution was prepared to 10nM by pre-mixing 10ul of Qtracker™ component A and component B in a 1.5ml tube and incubating for 5 minutes at room temperature. 2ml of fresh complete DMEM was added to the tube and mixed by vortexing for 30 seconds. Trypsinised cells at a concentration of 5×10^6 /ml were added to the labelling solution and incubated for 45-60 minutes at 37°C. Cells were washed twice in fresh complete DMEM by centrifugation. Labelled cells were reseeded into culture flasks and imaged using spinning disk confocal microscopy.

2.5.2 Vybrant™ DiD labelling

MetBo2 cells were labelled using Vybrant™ DiD cell labelling solutions (DiD Dye). Cells were suspended at a density of 1×10^6 /ml in serum-free DMEM. Cell-labelling solution was added at 5µl/ml of cell suspension and mixed by gentle pipetting. Cells were incubated at 37°C for 15 minutes and washed three times in fresh DMEM using centrifugation at 1500rpm for 5 minutes and resuspended in fresh complete DMEM. Labelled cells were seeded and imaged using spinning disk confocal microscopy.

2.5.3 Selection of mCherry⁺ MetBo2

To establish a mCherry⁺ MetBo2 cell line, cells were selected *in vivo* through rounds of injection and recovery to prevent immune rejection of the cells.

2.5.3.1 Fluorescence-activated cell sorting (FACS) of mCherry⁺ cells

mCherry⁺ MetBo2 and unlabelled MetBo2 cells were trypsinised and resuspended in 60×10^6 cells/ml in FACS buffer (1% BSA in PBS). Cells were sorted on the BD FACS Area Fusion using the unlabeled MetBo2 cells as a negative control. mCherry^{high} and mCherry^{int} cells were collected and reseeded in culture flasks.

2.5.3.2 Isolation of tumour cells from the bone

Tumour cells were isolated from metastatic bones by grinding the bone in a sterile mortar and pestle. Bone pieces were washed in fresh DMEM and filtered through a 70µm cell strainer. The cell suspension was centrifuged and the pellet resuspended in

fresh complete DMEM in a T25 flask. Once seeded, tumour cells were selected using antibiotic selection in 40µg/ml hygromycin.

2.6 *In vitro* assays

2.6.1 *In vitro* chemotherapy preparation

Paclitaxel was dissolved in Dimethyl sulfoxide (DMSO) at 50mg/ml and diluted in PBS to a stock concentration of 1mM. Doxorubicin was dissolved sterile water at 50mg/ml and diluted in PBS to a stock concentration of 1mM. Both were stored at -20°C and were diluted to the appropriate concentration in fresh culture medium as required.

2.6.2 Generation of conditioned media

Cells were grown to approximately 70-80% confluency. The culture medium was replaced with fresh culture medium and collected 24 hours afterwards. Conditioned medium was filtered using at 0.22µm filter and diluted 1/3 in fresh complete media before being used in the assay.

2.6.3 Proliferation assays

2.6.3.1 Indirect transwell assays

A transwell system was used for indirect co-culture proliferation assays. Cells were seeded at 1000 cells/well in a 24 well plate and effector cells were seeded at the appropriate ratio in 0.4µm-pore transwells in a separate plate. Fresh media only was added to control transwells. After 24 hours, the transwell inserts were transferred to the wells containing the tumour cells and chemotherapy added at appropriate concentrations. An MTT assay was performed after 3-5 days to measure cell growth.

2.6.3.2 Direct co-culture

For direct co-culture assays, 500 tumour cells/well and stromal cells were seeded together at appropriate ratios tumour:stromal cell ratio (1:1, 1:2, 1:4, 1:8) in a 48-well plate. After 24 hours, chemotherapy was added to the wells at appropriate concentrations. After 3 days, tumour cells growth was measured using a luciferase assay.

2.6.3.3 MTT assay

Media was replaced with 1ml/well of fresh DMEM containing 250µg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated for 1 hour at 37°C. Media was then removed and the cells washed in PBS before being dissolved in 100µl DMSO/well. 25µl from each well was transferred to a 96-well plate and the intensity measured using a spectrophotometer at optical density (OD) 540nm.

2.6.3.4 Luciferase assay

Luciferase assays were carried out as per manufacturer instructions. Briefly, culture medium was removed and cells washed in PBS solution. 100µl cell culture lysis buffer (1x diluted in water) was added to each well and shaken for 40 minutes at room temperature. 20µl of cell lysate from each well was transferred to a 96 well plate and the luminescence was measured using the CLARIOstar plus plate reader (BMG LabTech).

2.6.4 Migration assay

The transwell migration assay system was used to measure migration. Chemoattractant cells were seeded in 24 well plates at appropriate concentration and grown to approx. 80% confluency. Once confluent, media was replaced with fresh culture medium containing 10% FBS. Fresh media was added to control wells. Chemoattractant conditioned medium was also added as a variable. Migratory cells were seeded in 8.0µm-pore transwell inserts in 5% FBS culture media, and put into the wells. Cells were left to migrate for the appropriate time in the incubator.

To stain for migrated cells, the media was removed from the transwells, washed in PBS and cells fixed in 10% formalin for 20 minutes. Cells remaining in the transwell were removed using a cotton-tipped applicator. Cells were stained for 30 minutes in 0.05% crystal violet dissolved in water before being washed three times in water. Transwells were dried and imaged using a brightfield microscope.

2.6.5 Cell immunostaining

Cells were cultured in 48-well plates on glass coverslips that had previously been coated in poly-d-lysine for 1 hour before being washed in fresh culture medium and sterilized with 70% ethanol and UV. For staining, cells were washed three times in PBS for 1-2 minutes each, blocked for 1 hour in 10% normal goat serum (NGS) in PBS.

Primary antibody (mouse anti- α SMA Sigma A2547 lot: 076M47894V 1/400) was added at room temperature for 1 hour in 10% NGS. Cells were washed three times in PBS for 1-2 minutes each and incubated for 2 hours in secondary antibodies (goat anti-mouse IgG2a biotinylated A90-207B Bethyl laboratories 1/200) at room temperature. Streptavidin-AF488 (Invitrogen S32354) was added if required at 1/200 in PBS for 1 hour. Cells were stained with DAPI, washed in water and mounted onto slides. Slides were scanned using the AxioScan slide scanner (Zeiss).

2.7 Mouse studies

2.7.1 Ethics statement

All procedures involving mice were conducted in accordance with the UK Home Office regulations under the UK Animal Scientific Procedures Act (1986) (HO license number PPL P57A3693F, PIL IEF230586), and the institutional standards of the University of Edinburgh. Procedures and experiments concerning mice were approved by the University of Edinburgh Veterinary Services.

2.7.2 Mouse strains and breeding

Mice were housed and bred under standard conditions of care to generate the required lines in house. Colonies used throughout the project were maintained by members of the lab, including myself, Dr Carmen Huesa, Dr Galadrielle Biver, Dr Xue-Feng Li and Ruoya Ma.

FVB/N mice were obtained from Jackson Laboratories and were bred in house. Nestin-cre^{ERT2}-iDTR-GFP-nude mice were generated in house by crossing of C57BL/6-Tg(Nes-cre/ERT2)KEisc/, C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J (iDTR), C57BL/6-Tg(Nes-EGFP), and Foxn1nu/Crl. SP7-GFP/Cre/iDTR nude mice were generated by the mating of B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J, C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J, Foxn1nu/Crl. F-MacGreen mice were generated by crossing B6.Cg-Tg(Csf1r-EGFP)1Hume/J mice with FVB/N mice for 8 generations. FRag2 mice were generated by backcrossing the B6(Cg)-Rag2^{tm1.1cgn}/J mice with FVB mice for at least 8 generations. CD169-DTR-nude mice were generated by crossing of Siglec1^{tm1(HBEGF)Mka} and Foxn1nu/Crl mice. FMV mice were previously generated by crossing Tg(Csf1r-Mer-iCre-Mer)1Jwp transgenic mouse with Vegfa^{flox/flox} mice.

2.7.3 Breast cancer models

2.7.3.1 Bone metastasis assay

In the experimental model of breast cancer bone metastasis, 1.5×10^5 cells in 100 μ l sterile PBS were injected via intracardiac injection into 3-5 week old female mice, or 2×10^5 cells via intrailiac artery injection of 6-8 week old female mice under general anaesthesia (inhalational isoflurane in 100% oxygen, 1.5-2dm³/min). Intrailiac injection of cancer cells was performed as previously described⁸⁸. Briefly, a small incision was made in the lower right abdomen, and the fatty tissues pushed outward to expose the iliac vessels and nerves. Tumour cells were slowly injected in 100 μ l PBS at 2×10^6 cells/ml into the iliac artery using a 31G insulin syringe. A cotton swab was used to stop bleeding before being sutured to close the wound. Surgical guidelines including maintenance and monitoring of temperature were followed at all times. Bone metastasis growth was monitored using bioluminescent imaging.

2.7.3.2 Mammary fat pad tumours

In an experimental model of primary breast cancer, 1×10^6 cells in 100 μ l sterile PBS were injected percutaneously into the mammary fat pad (MFP) of 3-5 week old mice. Tumour growth was monitored using calipers to measure the length and width of the tumour. Tumour volume (mm³) was calculated where $\text{volume} = 1/2(\text{length} \times \text{width}^2)$.

2.7.4 Bioluminescent imaging (BLI)

D-luciferin (potassium salt) (100 μ l of 10mg/ml in PBS) was injected intraperitoneally into tumour-bearing mice. Bioluminescence from luciferase-expressing tumour cells was imaged using the Photon Imager Optima (Biospace Lab) under general anaesthesia (inhalational isoflurane in 100% oxygen, 1.5-2dm³/min). Photon counts (photon/second/cm²/sr) in the bone areas were quantified by image analysis software (M3 vision, Biospace Lab). To do this, the bioluminescent images were annotated with region of interests (ROI) at the relevant sites of the metastasis to quantify the bioluminescent signal within that area. A consistent size of ROI was used across experiments to ensure standardization of the measurements and quantification.

2.7.5 Chemotherapy treatment

Paclitaxel was prepared in sterile DMSO at a stock concentration of 5mg/ml. Doxorubicin was prepared in sterile PBS at a stock concentration of 10mg/ml. Both

were stored at -20°C and diluted in sterile PBS to a concentration of 1mg/ml in and 2mg/ml, respectively, for *in vivo* injection.

After tumour detection, mice were treated with chemotherapy every 5 days (day 0, 5 and 10) via intraperitoneal injection of 100µl chemotherapy (paclitaxel 10mg/kg; doxorubicin 5mg/kg) and euthanized on day 15.

2.7.6 Macrophage depletion

2.7.6.1 Liposome clodronate

Macrophages were depleted by liposome clodronate (Liposoma) administered via intravenous injection (150µl/20g mouse) at the times specified. Control liposomes were used for non-depleted control mice. Macrophage depletion was tested using flow cytometry and whole mount staining.

2.7.6.2 CSF1R inhibition

A CSF1R inhibitor, BLZ-945, was obtained from our Novartis and dissolved in 20% 2-hydroxypropyl-beta-cyclodextrin (prepared in ddH₂O) to a final concentration of 40mg/ml. BLZ solution was administered daily at 200mg/kg via oral gavage. Solvent only was provided as control.

2.7.6.3 CD169⁺ macrophage depletion

Bone resident macrophages were depleted using the CD169-DTR transgenic mice. Mice were administered with diphtheria toxin (DT) (or mutated Glu⁵²-diphtheria toxin (Glu⁵²-DT), containing a glycine to glutamic acid substitution mutation at position 52, for control mice) (1µg/kg) via intraperitoneal injection twice/week.

DT and Glu⁵²-DT was prepared in 5% sucrose and 1mg/ml BSA in PBS at a stock concentration of 2mg/ml. DT and Glu⁵²-DT was diluted in PBS to the required concentration for *in vivo* injections.

2.7.7 Nestin depletion

Nestin-cre^{ERT2}-iDTR-GFP mice were used to deplete the nestin⁺ cells as described previously¹⁷¹. Mice were administered with tamoxifen (143mg/kg) via intraperitoneal injection two times, every other day and then DT or Glu⁵²DT (4µg/kg) intraperitoneally every 2 days after the last tamoxifen injection.

Tamoxifen was prepared by diluting in 100% ethanol at a stock concentration of 200mg/ml and heated to 60°C until clear and stored at -20°C. For *in vivo* injection, tamoxifen was diluted in sterile corn oil to appropriate concentration at 37°C.

2.7.8 Osteoblast depletion

SP7-GFP-Cre-iDTR mice were removed from doxycycline diet 7-10 days before depletion. DT or Glu52-DT (25mg/kg) was administered daily via intraperitoneal injection¹⁷¹.

2.7.9 CD31 blood vessel labelling

To label the blood vessels, anti-CD31-AF647 antibody (Biolegend 102416 clone 390) was diluted in PBS to a concentration of 10µg/100µl (20µl in 180µl) and intravenously injected into the tail vein approx. 15 minutes before being culled.

2.7.10 Perfusion-fixation

Perfusion of PBS under terminal anesthesia (inhalational isoflurane in 100% oxygen, 1.5-2dm³/min) was used to remove the blood from the circulation. Briefly, the chest cavity was opened to expose the heart in which a cut was made in the right atrium. Sterile PBS was slowly injected into the left ventricle to flush blood and excess cells from the vasculature. This was repeated with 4% paraformaldehyde (PFA) if tissues required fixative perfusion. After dissection, tissues were fixed in 4% PFA for 30 minutes or overnight for whole mount staining and paraffin embedding, respectively.

2.8 Immunohistochemistry and immunofluorescent staining

2.8.1 Fixation and tissue processing

Tissues were fixed in 4% PFA overnight at 4°C and decalcified in 0.5M EDTA at pH7.4 for approx. 10 days changing EDTA every 3-5 days. Samples were dehydrated in increasing alcohol and embedded in paraffin wax before being cut into 10-15µm longitudinal sections using a microtome.

2.8.2 Immunofluorescent slide staining

Sections were incubated overnight at 55°C to melt wax and enhance adhesion to slide. Sections were dewaxed in histoclear two times for 5 minutes each before being

rehydrated in decreasing concentrations of ethanol (100% 100% 95% 80% 70% 50% for 2 minutes each). To reduce autofluorescence when required, samples were incubated in 0.2% sudan black diluted in 70% ethanol for 10 minutes between 70% and 50% ethanol. Antigen retrieval was performed by incubating the slides for either 5 minutes in boiling 1x citrate buffer at 30% power in a microwave, or at 55°C overnight depending on the primary antibody. Slides were then washed three times in PBS. Samples were permeabilised in 0.3% triton x-100 for 10 minutes and blocked in 10% NGS for 1 hour before being washed three times in PBS. Samples were incubated in primary antibodies overnight at 4°C in 10% NGS (mouse anti- α SMA Sigma A2547 lot: 076M47894V 1/400; rabbit anti-FSP abcam ab197896 lot: GR225386-11 1/200; rabbit anti-FAP abcam ab53066 lot: GR304998-11 1/200; rabbit anti-CD146 abcam ab75769 lot: GR208953-30 1/200; rabbit anti-sp7 Santa Cruz Sc-22536 R lot: B0416 1/100; rabbit anti-Iba1 abcam ab178846 lot: EPR16588 1/500; rabbit anti-Ki67 abcam ab15580 lot: GR233420-1 1/200; rat anti-Ki67 Invitrogen 14-5698-82 lot: 4304318 1/200; rabbit anti-CC3 CST 9664S lot: 21 1/200). The slides were washed three times in PBS before being stained with secondary antibodies at 1/200 for 2 hours at room temperature (donkey anti-rabbit Invitrogen AF555 A31572 lot: 1945911; goat anti-mouse IgG2a biotinylated A90-207B Bethyl laboratories; donkey anti-rat 488 Life Technologies A21208 lot: 1789917). Secondary only controls were performed for each secondary antibody. For biotinylated secondary antibodies, slides were also stained with streptavidin-Alexa Fluor488 (AF488) (Invitrogen S32354) for 1 hour at 1/200. Slides were washed once in PBS and two times in water before being stained with 10ug/ml DAPI for 5 minutes, washed three times in water and mounted using VectaShield®. Samples were imaged using AxioScan slidescanner.

2.8.3 H&E staining

Slides were incubated overnight at 55°C to melt wax and enhance adhesion of section to the slide. Sections were dewaxed in xylene two times for 5 minutes each before being rehydrated in decreasing concentrations of ethanol (100% 100% 95% 80% 70% for 20 seconds each). Slides were washed for 2 minutes in running water, stained by haematoxylin for 5 minutes and washed again for 20 seconds in running water. Slides were differentiated in 1% acid-alcohol for 5 seconds maximum and transferred to Scott's tap water substitute for 2 minutes until the tissue sections turned blue. Slides were stained in eosin solution for 30 seconds, and washed in running water for 20 seconds. Slides were then de-hydrated in increasing concentrations of ethanol for 20

seconds each and placed in xylene two times for 5 minutes each. Stained slides were mounted with Pertex® and a glass cover slip.

2.8.4 Whole/half mount imaging

Whole mount and half mount imaging was performed as previous³⁷. Bones were fixed in 4% PFA for 30 minutes and washed in PBS three times. Long bones were cryopreserved in 15% sucrose for 2 days at 4°C before being embedded in Optical Cutting Temperature compound (OCT) compound using a dry ice/ethanol bath. Long bones were shaved longitudinally using a cryostat to expose the bone marrow. The OCT was melted at room temperature and washed three times in PBS, before performing immunostaining.

Bones were permeabilised and blocked in 0.1-0.5% triton x-100 and 5% NGS overnight at 4°C. Samples were washed three times in PBS and incubated with primary antibodies in 20% NGS for 2 days at 4°C (mouse anti- α SMA Sigma A2547 lot: 076M47894V 1/400; rabbit anti-mCherry abcam ab167453 lot: GR3209879-3 1/200; mouse CD204 PE-conjugated antibody R&D FAB1797P; biotin rat anti-mouse IL4R BD Bioscience 552508). Bones were washed three times in PBS and incubated with the secondary antibody (goat anti-mouse IgG2a biotinylated A90-207B Bethyl laboratories) for 2 hours, washed three times in PBS and stained with streptavidin (AF488 (Invitrogen S32354) and AF647 (Invitrogen S21374) at 1/200 for 2 hours if biotinylated secondary antibody was used. Finally, samples were washed in PBS, stained with DAPI (10ug/ml) and washed three times in PBS.

Samples were imaged using a CSU-W1 spinning disk confocal (3i, Yokogawa) with a 20x water dipping objective. Images were analysed using SlideBook (3i).

2.8.5 Tissue clearance

Bones were collected from perfused-fixed mice and fixed in 4% PFA at 4°C overnight, washed and stained as described in the whole mount staining. Tissue clearance was optimized using different techniques using benzyl alcohol:benzyl benzoate (BA:BB), benzyl benzoate:polyethylene glycol (BB-PEG) and RapiClear1.52®.

2.8.5.1 BA:BB

For clearance using BA:BB¹⁷², bones were fixed in 4% PFA at 4°C overnight, washed and stained as described for whole mount staining. Bones were decalcified in 20% EDTA pH7.5 for 4 days at 37°C with constant shake. Samples were rehydrated in increasing Tert-butanol (30%, 50%, 70%, 80%, 96%, diluted in ddH₂O) for 30 minutes each and two times in 100% for 1 hour each at room temperature. The pH of tert-butanol was adjusted to above 9.5 with 3% triethanolamine. Samples were transferred into BA:BB at a concentration of 1:2 for final clearing and mounting. pH of BA:BB was also adjusted to above 9.5 using 3% triethanolamine.

2.8.5.2 BB:PEG

For clearance using BB:PEG¹⁷³, bones were fixed in 4% PFA at 4°C overnight, decalcified in 20% EDTA pH7.5 at 37°C for 4 days and washed in water for at least 30 minutes to elute excessive EDTA. Samples were then stained as described for whole mount staining. Samples were decolourised using quadrol for 2 days and 5% ammonium at 37°C in a shaker. Staining was performed as described in the whole mount staining. Samples were delipidated in tert-butanol solutions (30% for 4 hours, 50% for 6 hours, 70% for 1 day) and dehydrated in tert-butanol-PEG for 2 days with a daily change. Finally samples were immersed in BB-PEG at 37°C for at least 1 day for clearing and mounting.

2.8.5.3 RapiClear 1.52®

For clearance using RapiClear1.52®¹⁷⁴, samples were fixed in 4% PFA at 4°C overnight. Staining was performed as described for whole mount staining. Samples were immersed in RapiClear 1.52® heated to 37°C to improve penetration of the tissue. Clearance occurred overnight. For imaging, bones were mounted in an imaging chamber composed of the sample immersed in RapiClear1.52® between two coverslips separated by an iSpacer®. Images were acquired using the a 20x lens on the multimodal Imaging Platform Dragonfly (Andor technologies) and analysed using Imaris v9.0.

2.9 Flow cytometry

For flow cytometry analysis of *in vivo* populations, bones were extracted and cleaned of muscle and connective tissue. To isolate the cells, bones were crushed using a mortar and pestle, and digested in DMEM with no serum, 50ng/ml liberase TL, 50ng/ml

liberase DL and 10ng/ml DNase at 37°C, 700rpm for 30 minutes. The suspension was filtered into FACS tubes. The remaining bone was washed in PBS, which was also filtered through the FACS tube. The sample was centrifuged at 1200rpm for 5 minutes at 4°C. To lyse the red blood cells, the pellet was resuspended in 2ml RBC lysis buffer (diluted 1/10 in water) for 10 minutes on ice. Samples were washed twice in FACS buffer (1% BSA in PBS) by centrifugation at 1200rpm for 5 minutes at 4°C and resuspending FACS buffer and the total cells counted.

Cultured cells to be analysed by flow cytometry were trypsinised and resuspended at 250×10^3 - 500×10^3 cells/2ml in FACS buffer (1% BSA in PBS). Cells were centrifuged and the supernatant removed. FACS antibodies were added to the relevant FACS tube for each panel. Macrophage polarization: IL4R-PerCP/Cy5.5 BD Biosciences 11B11; CD204-PE R&D 268318; PD1-PE/Dazzle BioLegend 29F.1A12; PDL1-PE/Cy7 eBioscience MIH5; Tim4-AF647 BioLegend RMT4-54; MHCII-A700 BioLegend M5/114.15.2; F4/80-BV650 BioLegend BM8; CD45-APC/Cy7 BioLegend; 30-F11. Myeloid panel: CD169-FITC BioLegend 3D6.112; IL4R BD BioSciences 11B11; CD204-PE R&D 268318; F4/80-PE/Dazzle BioLegend BM8; Ly6G-PE/Cy7 BioLegend 1A8; CCR2-APC R&D 475301; CD45-APC/Cy7 BioLegend 30-F11; Ly6C-BV510 BioLegend HK1.4; CD115-BV605 BioLegend AFS98; CD11b-BV711 BioLegend M1/70. Lymphoid panel: B220-FITC BioLegend RA3-6BR; CD4-PerCP/Cy5.5 BioLegend GK1.5; FOXP3-PE eBioscience FJK-16s; CD19-PE/Dazzle BioLegend 6D5; CD3-PE/Cy7 BioLegend 17A2; CD25-APC BioLegend PC61; CD45-APC/Cy7 BioLegend 30-F11; NK1.1-BV650 BioLegend PK135; CD8-AF700 BioLegend 53-6.7. Samples were incubated with the antibodies for 20 minutes in dark at 4°C. The sample was washed in FACS buffer by centrifuging for 5 minutes at 300g 4°C and resuspended at 60million cells/ml. Flow analysis was performed on a 6L LSR Fortessa (BD) and analysed using FlowJo V10 (BD).

2.10 Intravital imaging

2.10.1 Window design and manufacture

Windows were designed using computer aided design (CAD) software (Fusion CAD 360). Prototypes were 3D-printed and optimized on cadavers. Titanium windows were manufactured by ProtoLabs using fine-resolution direct metal printing built in 20µm layers with a standard finish.

2.10.2 Window insertion surgery

Femur windows and calvaria windows were surgically inserted onto the respective tissues for intravital imaging to live image cellular interactions. Surgery was performed under sterile conditions and with aseptic techniques. All surgical instruments and windows were sterilized by autoclaving. The mice were anaesthetized using 1.5-2dm³/min isoflurane in 100% oxygen and given buprenorphine (Vetergesic[®]) as an analgesic as per university guidelines. Eye ointment was applied to the eyes. The body temperature was monitored and maintained using a heat mat. Surgical procedures were performed under a surgical microscope (Leica).

To insert the calvaria window, the hair was removed using hair removal cream and the scalp swabbed with surgical scrub to sterilize the surgical area. A small incision was made using a scalpel and a small area of the skin removed to expose the skull.

Leftover membrane from the top of the head was scraped off using a scalpel.

VetBond[™] (3M) was applied to the edges of the incision to stick the skin to the skull and create a seal. Once dry, the window was glued to the skull using a mixture of superglue and dental cement with the edge of the incision being covered by the window. Additional dental cement was used to close any exposed areas of spaces that form the outer edge of the window. PBS was applied to the skull to prevent it from drying and a sterile coverslip inserted into the window and a circlip used to secure it in place.

The femur window was inserted as described previously¹⁷⁵. Briefly, the surgical site was prepared by removal of the hair and sterilization using surgical scrub. A 10mm longitudinal skin incision was made using a scalpel approaching the femur from the lateral side. The femur between the flexor and extensor muscles was exposed by blunt dissection using forceps. The U-shaped bar was inserted under the bone and the window inserted. The bar was secured using two nuts. A mixture of dental cement and superglue was used to secure the window to the mouse and seal the skin-window interface. The area surrounding the bone in the window was also filled with dental cement. The cortical bone was ground down with a micro-drill to gain optical access to the marrow cavity. The thinning of the bone was monitored under the microscope while performing the procedure so that a thin transparent periosteal layer of the bone was left intact. A coverslip was placed over the window and secured with a circlip. The musculature was re-adjusted to its original position with forceps to maintain their

function following the surgery. Mice were monitored during recovery and housed in standard conditions.

2.10.3 Image acquisition using confocal microscopy

Imaging was acquired using a CSU-W1 spinning disk microscope (3i, Yokogawa) and performed under university guidelines. The mice were anaesthetized using 1.5-2 dm³/min in 100% and maintained throughout the imaging. Eye ointment was applied to the eyes. Intravascular dyes were injected intravenously to label the vasculature (dextran). A custom-made imaging stage was used to stabilize the mouse by securing the window to the stage. A rectal thermometer was used to monitor temperature and a thigh/paw clip used to monitor oxygen saturation and pulse. A heat mat was used to maintain physiological temperature at 37°C. After imaging, the mouse was recovered and put back into housing cages until subsequent imaging.

Images were acquired up to 6 hours once a week or 3 hours/day up to 1 week. Vasculature maps, reference points and x,y co-ordinates were used to relocate the microscope to the same location each imaging session.

2.11 Statistical analysis

Statistical analysis was performed using Prism (GraphPad Inc.) Standard student's T-tests and analysis of variance (ANOVA) were used for two data sets and multiple data sets, respectively. P values below 0.05 were deemed to have statistical significance.

Chapter 3 Role of macrophages in bone metastatic seeding and colonisation of breast cancer

3.1 Introduction

Metastasis is an inefficient process. The majority of invasive cancer cells that enter the circulatory system and subsequently disseminate to secondary organs will perish. In order to ensure their survival at distant metastatic sites, tumour cells must therefore seed and colonise a metastatic niche, which provides essential survival factors.

Tumour cell seeding is therefore a key, rate-limiting step of the metastatic cascade¹⁷⁶.

There is now substantial evidence to demonstrate the significance of macrophage populations in tumour development and metastasis in the primary tumour^{130,177,178}.

However, the role of macrophages at the metastatic site, particularly bone metastasis, is less studied, but as important in overcoming the disease. Whilst it is evident that macrophages significantly contribute to the metastatic niche in the breast cancer lung metastasis, it remains to be elucidated whether they are involved in seeding and colonisation in the bone. We will therefore investigate this using an experimental model of breast cancer bone metastasis. We will first determine whether macrophages contribute to breast cancer cell colonisation by demonstrating the effect of macrophage depletion on early cell seeding and long term growth. We will subsequently define the mechanism that macrophages use to promote seeding. A distinct macrophage niche may exist. Macrophages within this niche may secrete chemoattractants to attract tumour cells, such as VEGFA observed in lung metastasis¹⁴⁵, or may alter the ECM and facilitate adhesion and seeding^{179–181} (figure 3.1.1).

Alternatively, macrophages may also modulate the already established endosteal and perivascular HSC niche to promote tumour cell seeding. As described previously in chapter 1, the bone metastatic niche overlaps with the HSC niches in both breast and prostate cancer cells^{182,183}. The perivascular and endosteal HSC niches, composed of MSCs/pericytes and osteoblasts, respectively, provide a critical supportive environment for the survival and maintenance of disseminated bone metastatic tumour cells. DTCs

seed to and colonise these niches by establishing molecular interactions with the stromal cells within these niches, including cadherins and gap junctions^{88,103}. Bone resident macrophages, known as osteomacs, are an important component of the HSC niche forming interactions with both the osteoblasts and MSCs. CD169⁺ macrophages are involved in retention of HSCs at nestin⁺ MSCs in the perivascular niche via the promotion of CXCL12 expression from MSCs¹⁸⁴. Similar effects were also observed at the endosteal niche where macrophage depletion in MAFIA mice or liposome clodronate shrinks the endosteal niche by reducing the number of osteoblasts resulting in downregulation of factors promoting HSC retention and HSC mobilisation⁸³. Together, these studies demonstrate an important macrophage-mediated role in maintaining both the endosteal and perivascular niche. Due to the similarities between the retention of HSCs and tumour cells in the niche, we propose that macrophage-mediated regulation of MSCs and osteoblasts, as well as the retention of HSCs to their niche observed in normal bone^{184,185}, may also occur in seeding of bone metastatic cells.

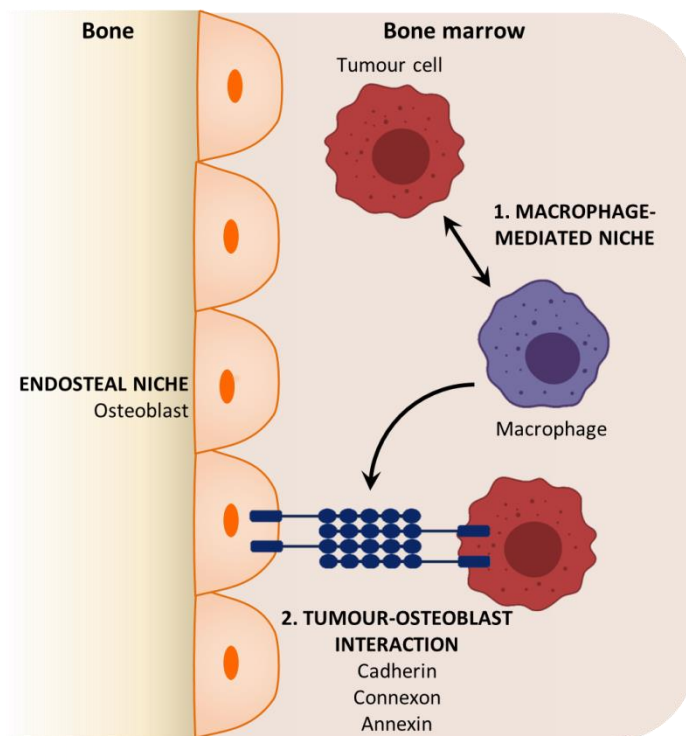


Figure 3.1.1: Role of macrophages during bone metastatic seeding and colonisation. Macrophages can promote seeding by directly forming a niche and secreting chemoattractants, or by mediating interactions at existing niches within the bone, including the endosteal and perivascular HSC niche.

We therefore aim to investigate whether macrophages provide a niche that directly facilitates seeding, or whether macrophages modulate the endosteal or perivascular niche. To achieve this, we will also develop imaging techniques to image the bone. The development of a novel intravital imaging platform will allow imaging of the tumour-stromal interactions in real-time, rather than a snapshot at a specific stage, permitting the dynamics and kinetics of the tumour cell migration and tumour-stromal interactions to be monitored. The development of a tissue clearance protocol will allow imaging of the bone in three dimensions. This will not only increase the accuracy of distance measurements, but also allow a holistic image of the entire bone.

3.2 Results

3.2.1 Effect of macrophage depletion on tumour cell seeding

The role of macrophages in breast cancer cell seeding to the bone was investigated using an *in vivo* seeding assay in which MetBo2 cells were injected into the intrailiac artery to establish bone metastasis (figure 3.2.1). Mice were treated with a single standard dose of liposome clodronate 24 hours before injection of MetBo2 cells to deplete macrophages during the seeding period. Tumour cell abundance and growth was monitored during early stage seeding and every 5 days in later tumour growth using bioluminescence imaging. The depletion efficiency of the single liposome clodronate dose was tested using flow cytometry of the bones collected from control and macrophage depleted mice at 48 hours of the experiment regime (figure 3.2.2a). The single dose of liposome clodronate results in a significant reduction (~25%) in the number of macrophages 3 days after liposome clodronate injection. This was confirmed using whole mount staining of control and macrophage depleted FMacgreen mice (figure 3.2.2b).

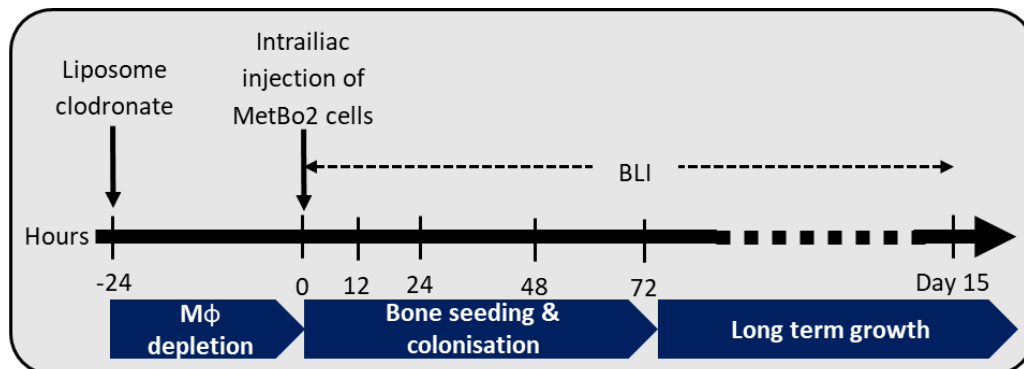


Figure 3.2.1: Effect of macrophage depletion on MetBo2 seeding. Experimental design: seeding assay in Macgreen (Csfr1-GFP) or FVB mice (BLI, bioluminescence imaging).

The effect of macrophage depletion on tumour cell seeding was monitored using bioluminescence imaging to determine the number of tumour cells in the bone. Mice were imaged immediately after intrailiac injection to establish a base line abundance of tumour cells reaching the bone after injection. Mice were subsequently imaged 2 days after injection to quantify the number of tumour cells persisting in the bone. Macrophage depletion during the seeding period significantly reduces the seeding efficiency of the MetBo2 cells measured by the BLI signal 2 days after injection (figure 3.2.3).

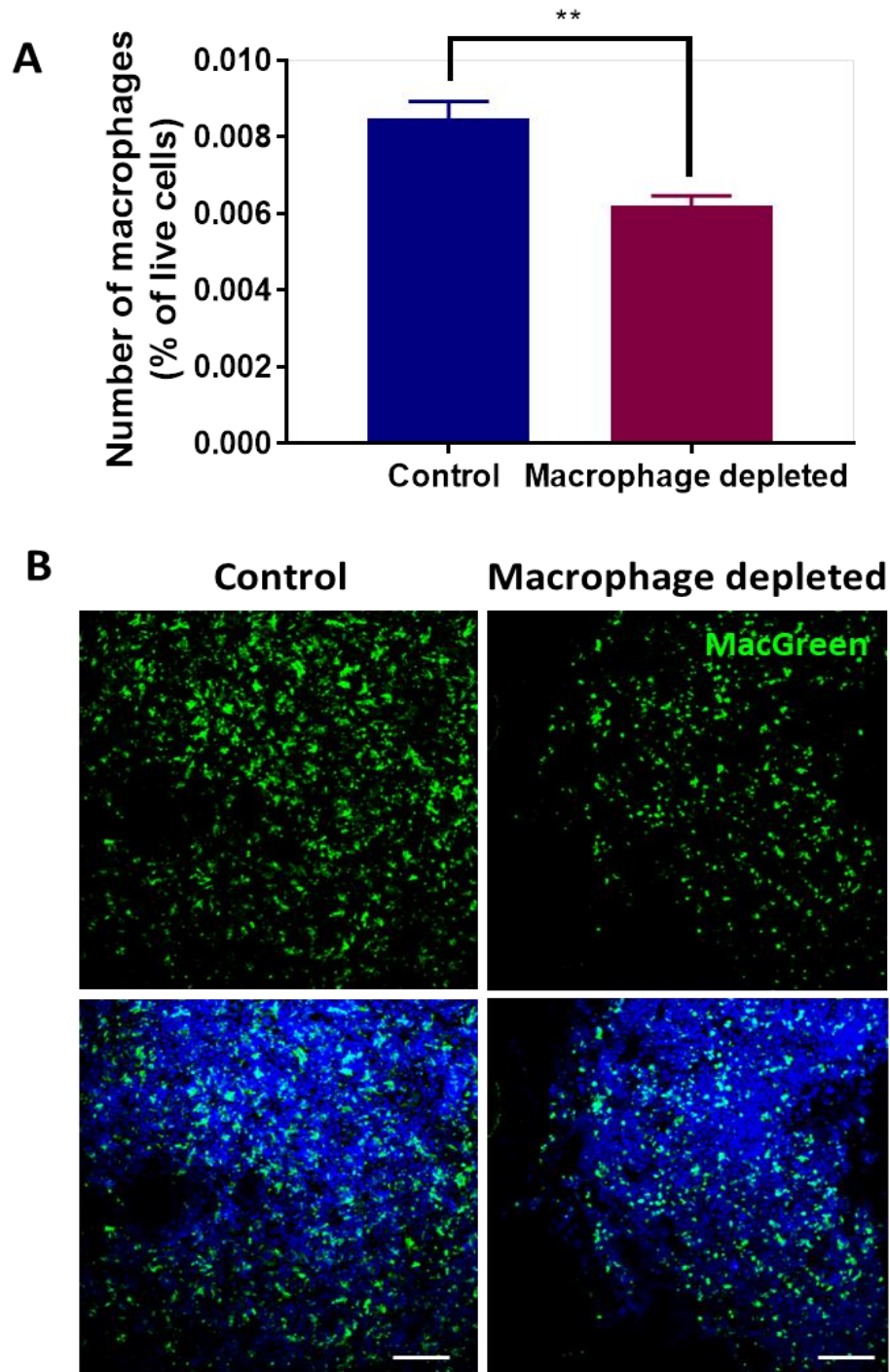


Figure 3.2.2: Macrophages are depleted after a single dose of liposome clodronate. A) Quantification of macrophages in control and macrophage depleted mice measured using flow cytometry analysis. Data shown are mean $\pm$ SEM (** $P < 0.005$, unpaired student's t-test, $n = 4-6$). **B)** Whole mount fluorescent image (maximum projection) of bone marrow collected at 48 hours after MetBo2 injection from control and macrophage-depleted MacGreen mice. Scale bar: 100 μ m.

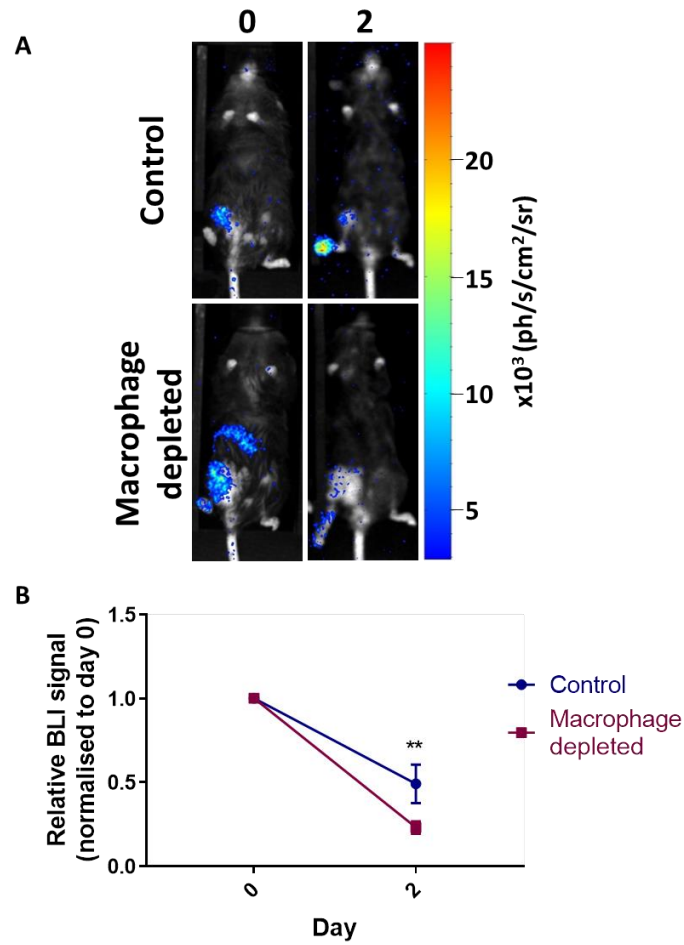


Figure 3.2.3: Macrophage depletion reduces the number of tumour cells in the bone during tumour cell seeding. A) Representative images of BLI signal at day 0 and 2. **B)** Quantification of the short term BLI signal immediately after and 2 days after MetBo2 injection. Data shown are mean $\pm$ SEM (** $p < 0.005$, two-way ANOVA, $n = 5-6$).

A similar experimental design was used to study the effect of macrophage depletion on long term growth where mice were imaged immediately after tumour cell injection to establish a base line of tumour cells reaching the bone, and imaged every 5 days to monitor tumour growth. BLI quantification demonstrates that tumour growth was significantly delayed in macrophage depleted mice compared to non-depleted controls (figure 3.2.4a, figure 3.2.4b). This resulted in a significantly increased survival of the macrophage depleted group where survival was measured as the time to humane endpoint (figure 3.2.4c).

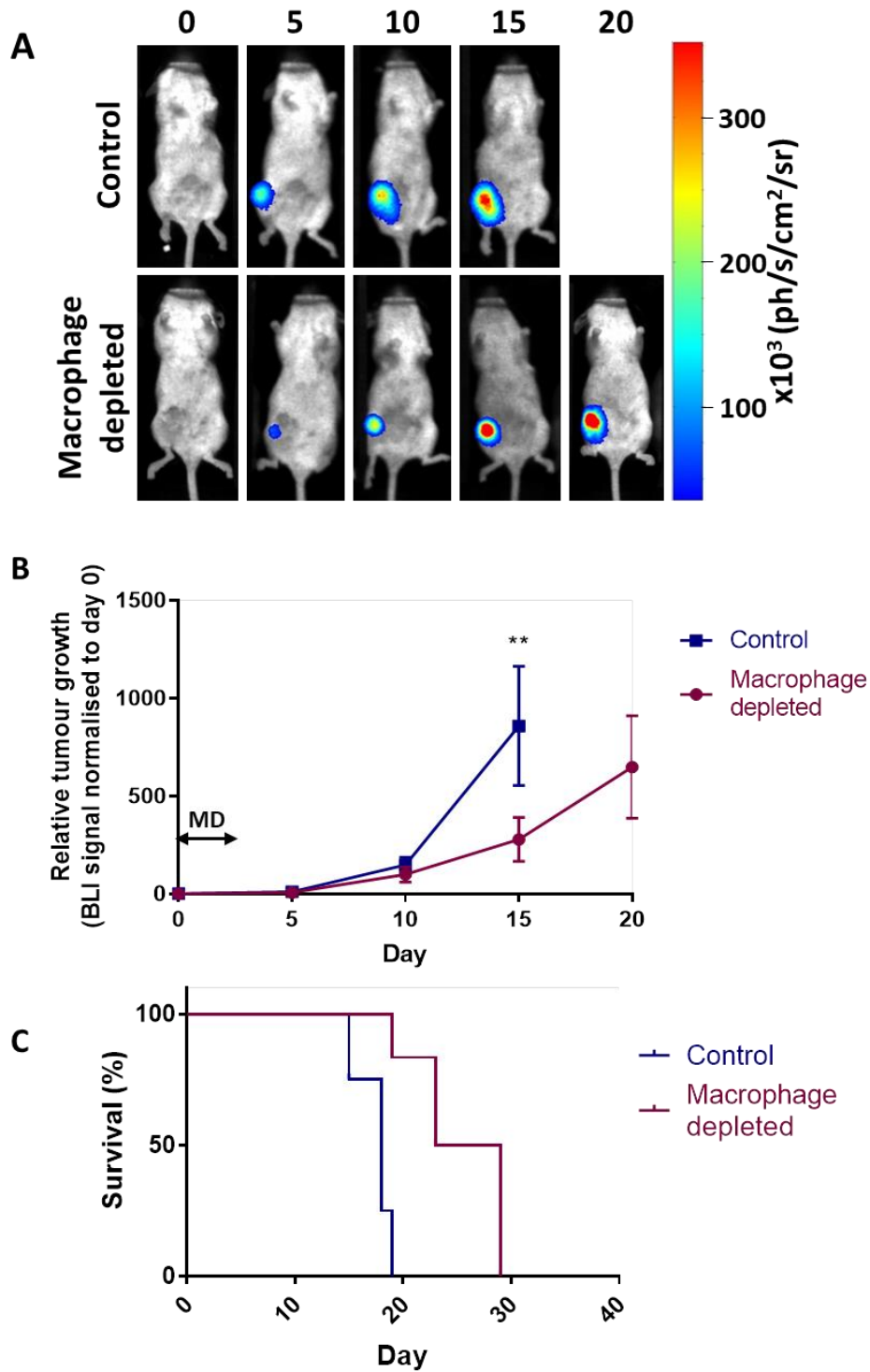


Figure 3.2.4: Macrophage depletion during seeding significantly delays long term bone metastatic growth and prolongs survival. A) Representative images of bioluminescence signal throughout tumour growth. **B)** Quantification of the long term bone metastatic growth in control mice and mice receiving macrophage depleted during seeding (MD, macrophage depletion) (** $p < 0.005$, two-way ANOVA, $n = 4-6$). **C)** Survival of mice in control and macrophage depleted mice measured as the number of days from intrailiac injection of tumour cells to humane endpoint. Data shown are mean $\pm$ SEM (** $p < 0.005$, Mantel-Cox test, $n = 4-6$).

3.2.2 Macrophage-derived VEGF does not play a role in tumour cell seeding

VEGFA has previously been demonstrated to play a role in macrophage-mediated seeding in breast cancer lung metastasis¹⁸⁶. To determine if VEGFA signalling plays a role in macrophage-mediated tumour seeding in bone metastasis, the seeding assay was performed in FMV mice. These were previously generated by crossing mice that express tamoxifen inducible Mer-iCre fusion protein driven by the *Csf1r* promoter with *Vegfa*^{flox/flox} mice (figure 3.2.5a)¹⁸⁷.

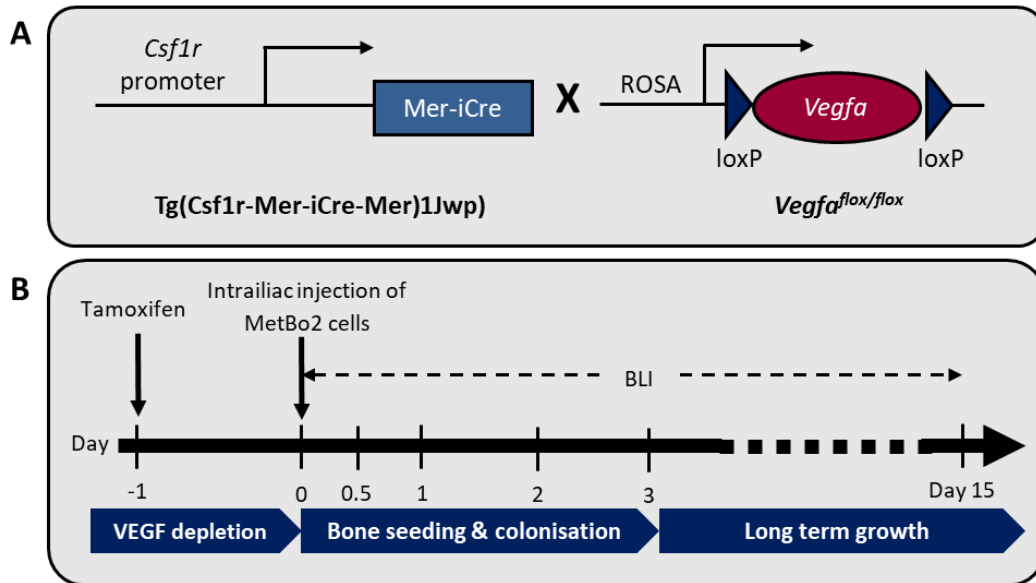


Figure 3.2.5: Effect of VEGF deficiency in macrophage cells during seeding. A) Genetic details of the FMV mouse model. **B)** Experimental design: experimental bone metastasis assay in FMV mice with macrophage-specific *Csf1r*-iCre-induced VEGF knockout *Cre*^{-/-} mice and *Cre*^{+/-} littermate controls.

The administration of tamoxifen therefore conditionally ablates *Vegfa* in *Csf1r*-expressing myeloid cells. Tamoxifen was administered one day before intrailiac injection of MetBo2 cells. Bone metastasis seeding and growth was again monitoring using bioluminescent imaging every 12-24 hours during seeding, and subsequently every 5 days during late stage growth (figure 3.2.5b). VEGFA depletion in macrophages during seeding had no effect on tumour cell seeding capacity (figure 3.2.6) or subsequent long term growth (figure 3.2.7).

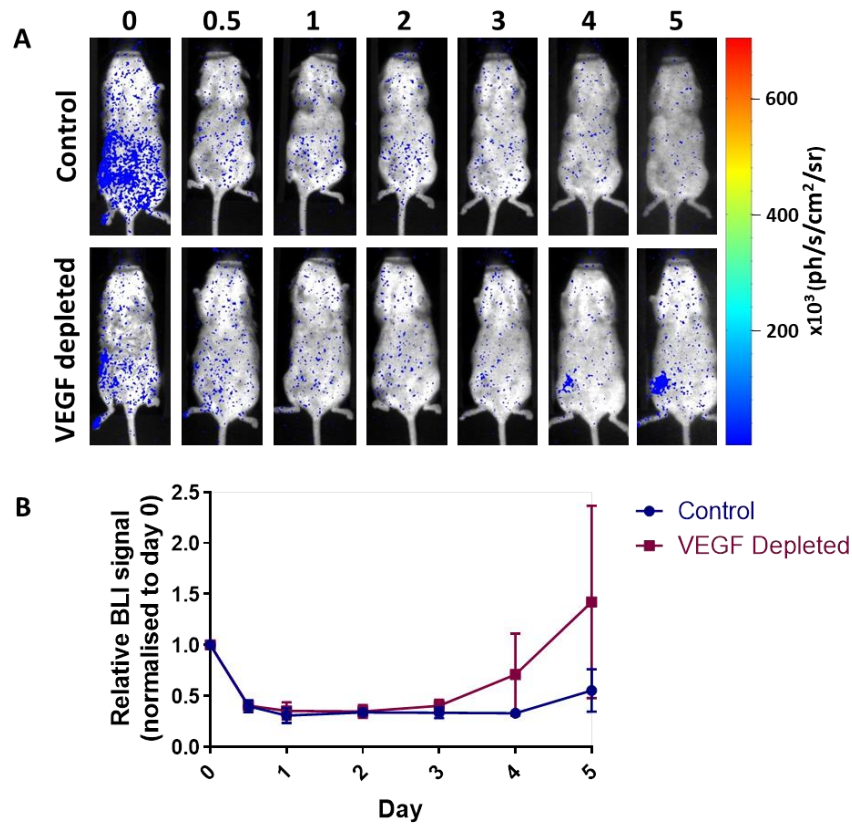


Figure 3.2.6: VEGF deficiency in macrophages during seeding has no significant effect on tumour cell number during seeding. A) Representative images of the BLI signal throughout tumour cell seeding. **B)** Quantification of the short term BLI signal during the first 5 days after MetBo2 injection. Data shown are mean $\pm$ SEM (n=3-4).

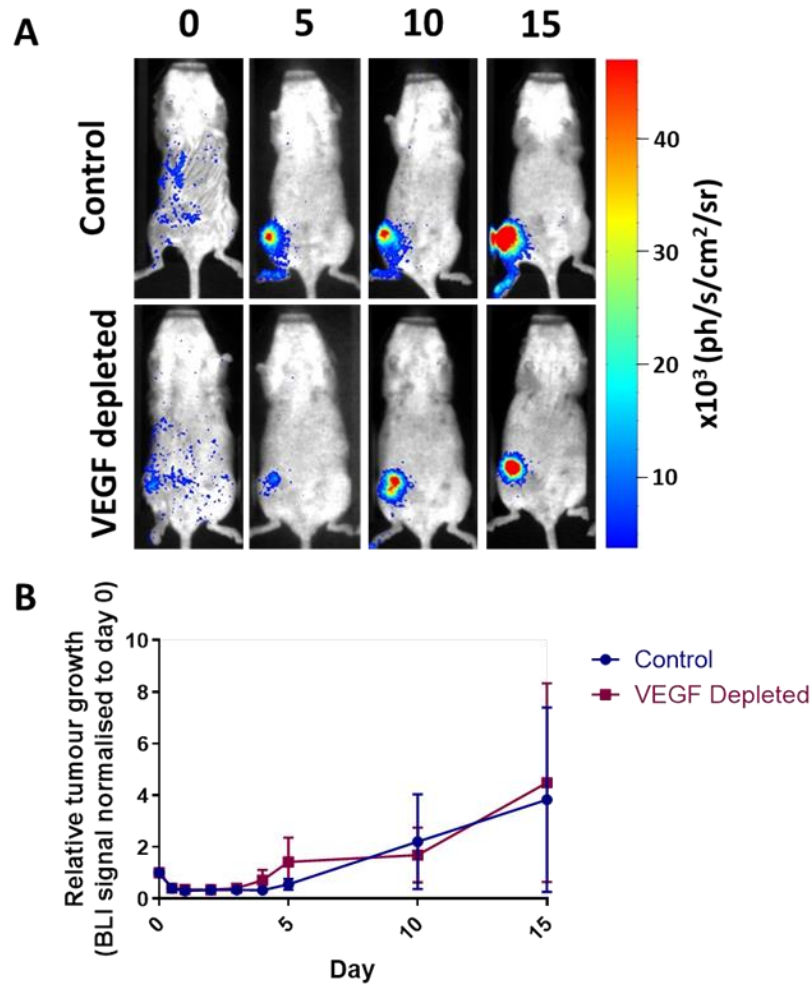


Figure 3.2.7: VEGF deficiency in macrophages during seeding has no significant effect on long term bone metastatic growth. A) Representative images of the BLI signal throughout bone metastatic growth in control and VEGF-depleted mice. **B)** Quantification of long term bone metastatic growth in control mice and mice with VEGF deficiency during seeding. Data shown are mean $\pm$ SEM (n=3-4).

3.2.3 Tumour cells appear to seed to the osteoblast-rich region

The location of tumour cells during seeding was analysed using whole mount imaging. It should be noted that the mCherry⁺ MetBo2 cells used for this experiment had already undergone selection as described below. Bones were collected from non-depleted mice at 48 hours after mCherry⁺ MetBo2 cells were implanted via intrailiac artery injection. Blood vessels were stained by intravenous injection of anti-CD31 antibody 10 minutes before culling. Whole mount imaging using confocal microscopy was used to identify the labelled tumour cells within the bone (figure 3.2.8a). The MetBo2 cells were located either within or around the blood vessels (figure 3.2.8b) suggesting tumour cells were extravasating. Furthermore, the majority (83.3%) of MetBo2 cells were found

around the blood vessels in the osteoblast-rich epiphysis region below the epiphysis plate in the 3 bones imaged. In contrast very few tumour cells were found in the bone marrow (figure 3.2.8c) indicating that the tumour cells were undergoing extravasation and seeding within the osteoblast-rich region, but requires confirmation using SP7-GFP mice.

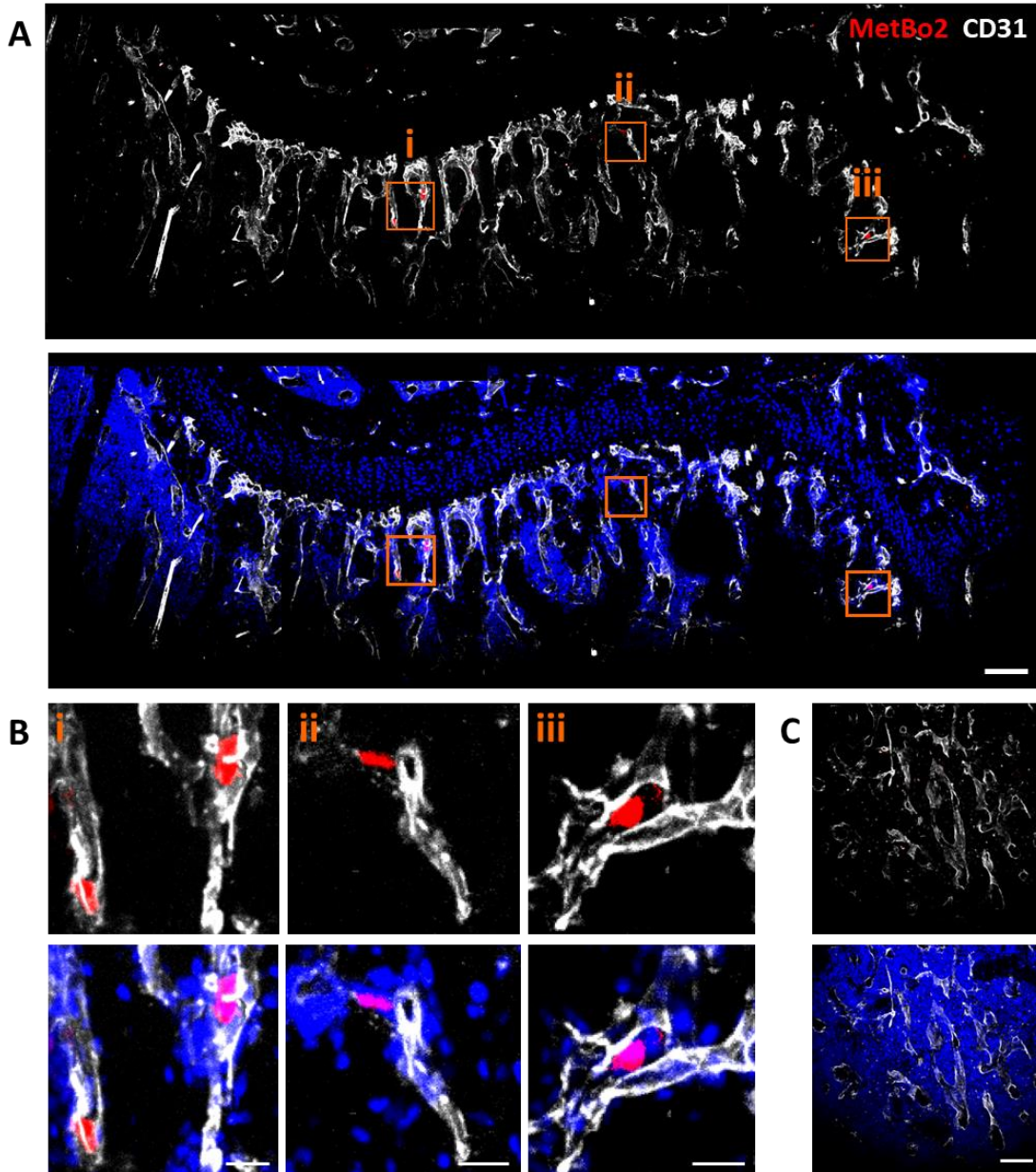


Figure 3.2.8: Breast cancer cells seed to the osteoblast-rich region of the bone marrow.
A) Whole mount fluorescent image (maximum projection) of the epiphysis region/growth plate 48 hours after mCherry⁺ MetBo2 injection stained for CD31 (top) and merged with DAPI (bottom). Scale bar: 100µm. **B)** Region of interests containing mCherry⁺ MetBo2 cells from (A). Scale bar: 20µm. **C)** Whole mount fluorescent image (maximum projection) of the bone marrow 48 hours after mCherry⁺ MetBo2 injection stained for CD31 (top) and merged with DAPI (bottom). Representative images of 3 bones collected from 3 separate mice are shown.

3.2.4 Establishing labelled MetBo2 cells

To further investigate the location of the tumour cells during seeding, we developed several imaging techniques, including intravital imaging and tissue clearance. In order to identify the tumour cells *in vivo*, MetBo2 cells were fluorescently labelled. Whilst mCherry⁺ MetBo2 cells had previously been established in the lab, there were concerns regarding immune rejection and therefore alternative labelling techniques were sought.

We first labelled the MetBo2 cells using Q-dot cell tracker, a fluorescent labelled nanocrystal that enters the cell cytoplasm. The MetBo2 cells were successfully labelled with the Q-dot tracker (figure 3.2.9a). Labelled-MetBo2 cells and unlabelled controls were implanted via intracardiac injection into FVB/N mice. After 24 hours, mice were injected with anti-CD31 labelling antibody before being culled. Imaging of whole mount bones was performed to identify the labelled MetBo2 cells in the bone marrow (figure 3.2.9b). Labelled MetBo2 cells were identified within and around the blood vessels of the marrow, which were not identified in the bones from mice injected with unlabelled tumour cells. To test the toxicity of the Q-dot cell tracker on the MetBo2 cells, proliferation assays were carried out on Q-dot labelled cells and labelled controls. The labelled cells were seeded into 6-well plates immediately after the labelling procedure. The labelling procedure was also performed on the unlabelled cells with sterile PBS being added instead of the Q-dot labelling solution. An MTT assay was carried out on the cells after 3 days. The Q-dot-labelled MetBo2 cells exhibited a significantly reduced cell viability compared to the unlabelled controls after only 2 days in culture (figure 3.2.9c). As the Q-dot tracker was toxic to the MetBo2 cells even after only 2 days, it rendered this labelling technique unsuitable for the *in vivo* seeding assay.

Subsequently, mCherry⁺ MetBo2 cells were successfully labelled with lipophilic dye, DiD dye and were imaged using confocal microscopy on day 1 and 3 after labelling (figure 3.2.10). On day 1, labelled cells exhibited a clear and defined staining, whereas the cells exhibiting a diffuse pattern of the DiD dye with many cells containing very low levels of the dye. This indicates that as the cells begin to divide, the DiD dye is distributed between the daughter cells and therefore over time the cells will lose the fluorescent label. This technique was therefore unreliable for *in vivo* imaging.

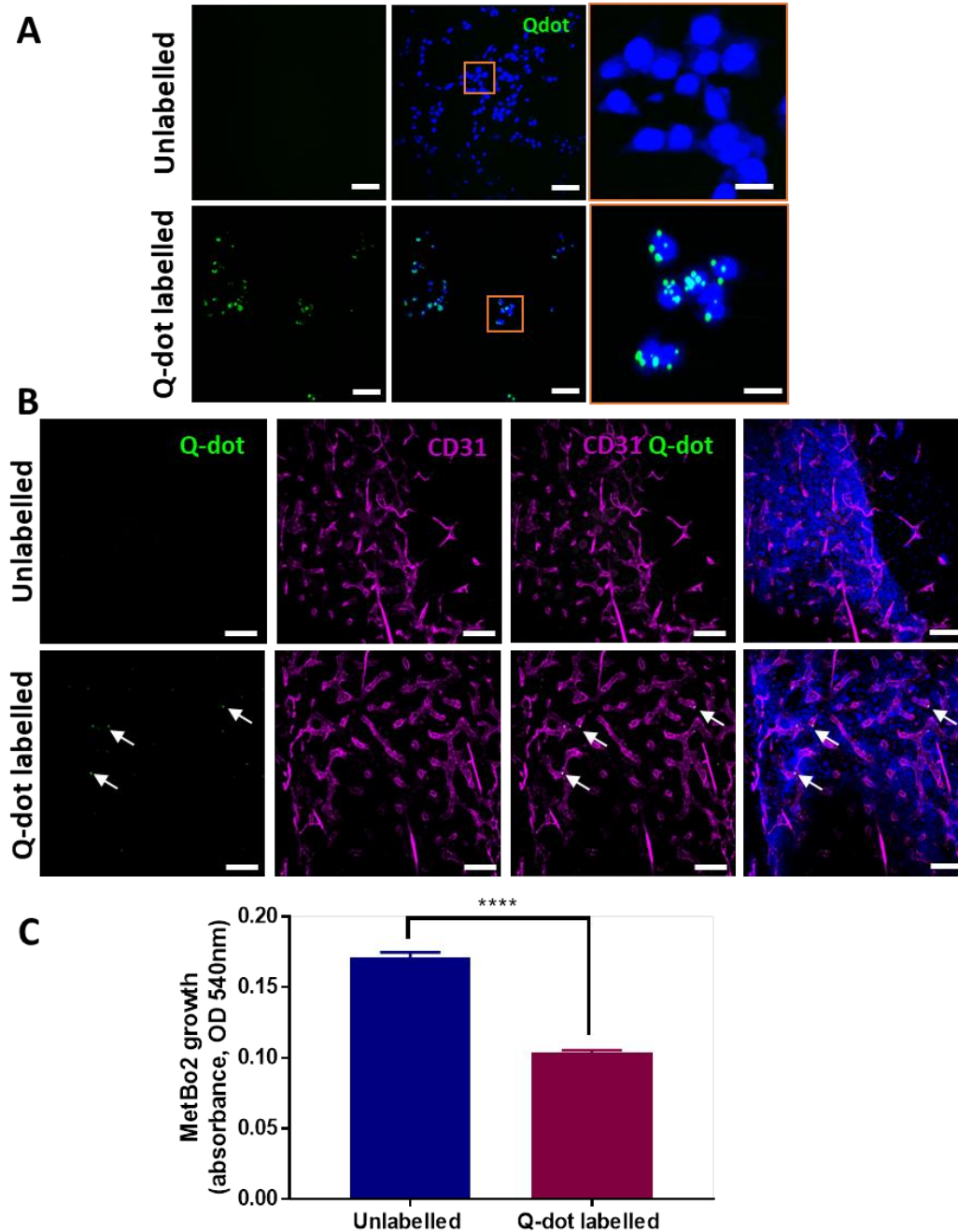


Figure 3.2.9: Q-dot labelled MetBo2 cells can be identified *in vivo*, but exhibit significant toxicity. **A)** Immunofluorescence images of unlabelled MetBo2 cells and MetBo2 cells labelled with Q-dot cell tracker (left) and merged with DAPI (centre). Scale bar: 100µm. Regions of interest are shown on the right. Scale bar: 25µm. **B)** Whole mount immunofluorescence images stained with Q-dot, CD31, Q-dot+CD31 and merged with DAPI (left-right) showing bone marrow in femurs collected from mice 48 hours after injection of unlabelled (top) and labelled (bottom) MetBo2 cells. White arrow: MetBo2 cells; scale bar: 100µm. **C)** Proliferation assay of unlabelled and q-dot labelled MetBo2 cells after 2 days in culture measured using an MTT. Data shown are mean±SEM (****p<0.0001, unpaired student's t-test, n=3).

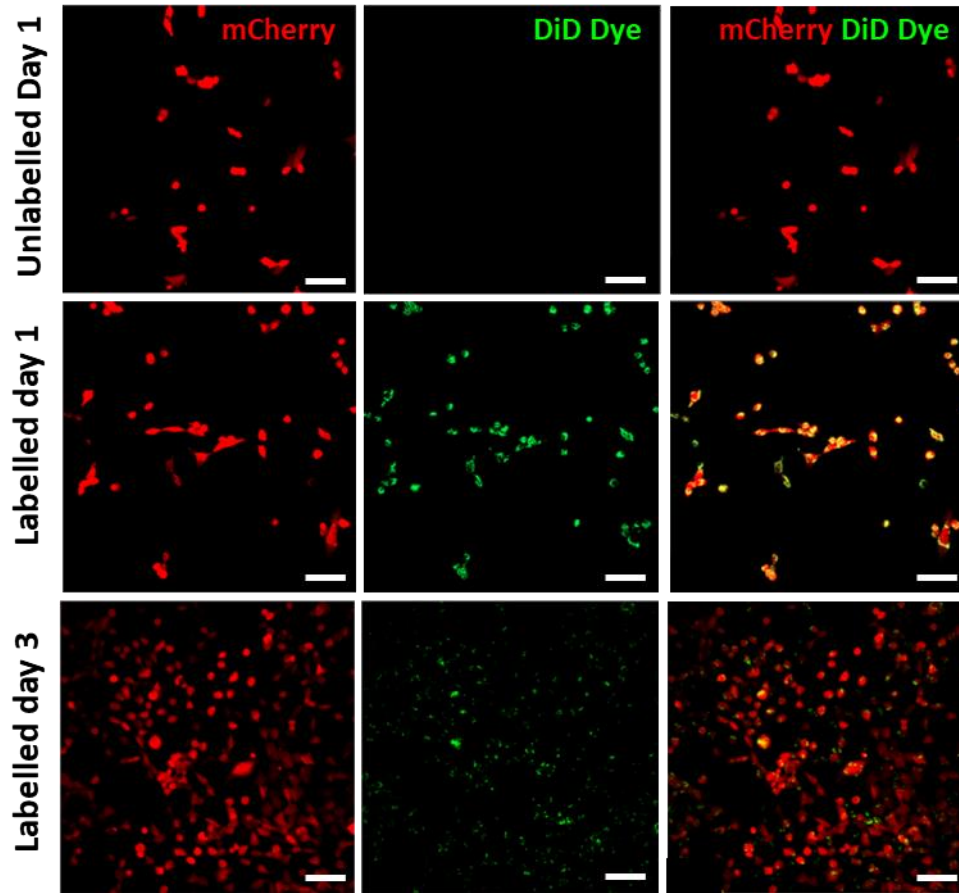


Figure 3.2.10: MetBo2 cells do not retain labelling with DiD Dye. Immunofluorescence images of unlabelled mCherry⁺ MetBo2 cells (top) and mCherry⁺ MetBo2 cells labelled with DiD lipophilic dye on day 1 (middle) and day 3 (bottom) after the labelling procedure. Scale bar: 100µm.

Finally, the mCherry⁺ MetBo2 cells were therefore used and selected through several rounds of *in vivo* selection to prevent immune rejection. MetBo2 cells were previously labelled and confirmed using both fluorescence imaging (figure 3.2.11a) and flow cytometry (figure 3.2.11b). The expression level of mCherry showed variation, and therefore the cells were sorted for the brightest population of cells to increase their visibility and therefore detection during *in vivo* imaging. The first round of selection was performed with the mcherry^{high} MetBo2 via subcutaneous injection to generate tumours in FVB/N mice. Tumour cells were isolated from established subcutaneous tumours and cultured *in vitro*. Sorting for mCherry^{high} cells was repeated before being intracardially injected into 5 FVB/N mice to establish bone metastasis. The bone metastatic growth was monitored using bioluminescent imaging.

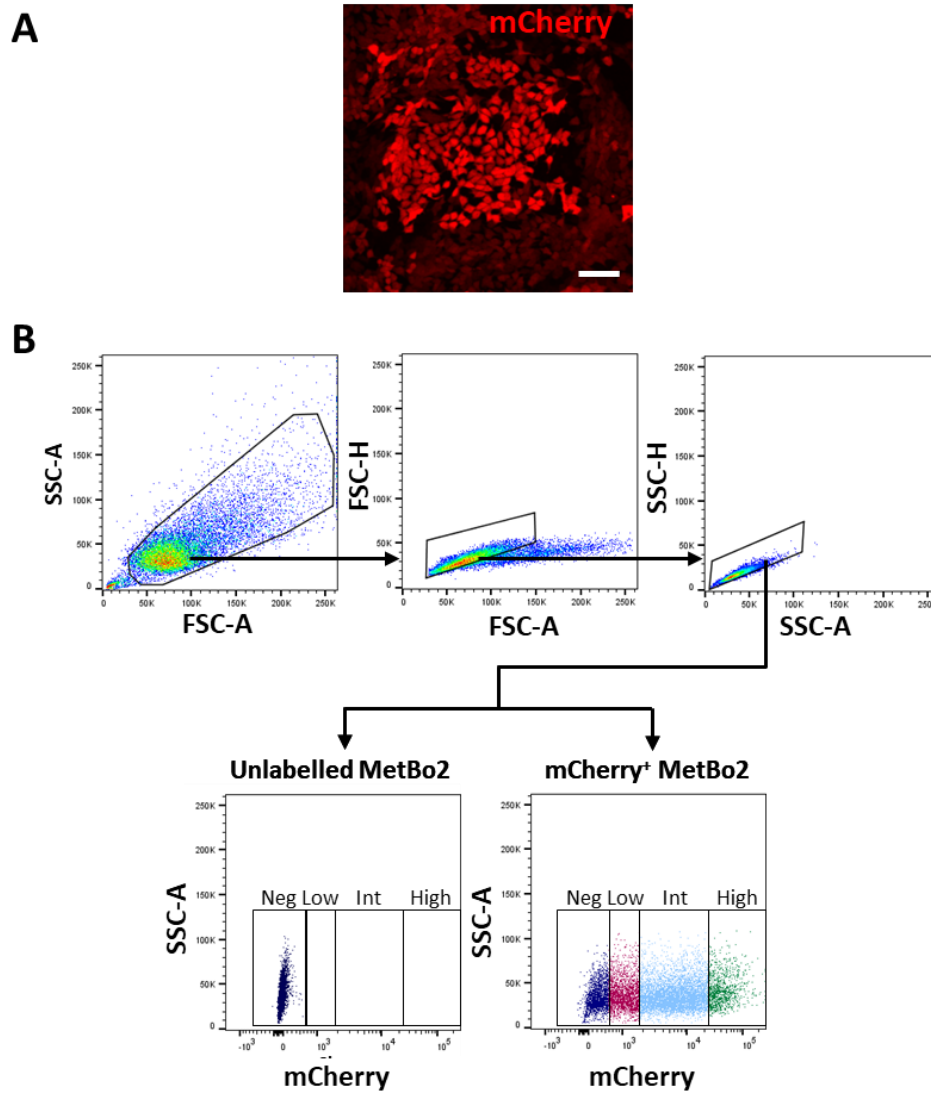


Figure 3.2.11: mCherry⁺ MetBo2 exhibit differential fluorescence levels of mCherry. **A)** Immunofluorescence image of mCherry⁺ MetBo2 cells *in vitro*. Scale bar: 100µm. Image shown is representative of 3 separate *in vitro* seedings of mCherry prepared for imaging. **B)** Gating strategy used to sort mCherry⁺ MetBo2 cells into mCherry negative, low, intermediate and high populations (n=1).

Only one mouse from the five injected established a tumour in the jaw, which grew for about 14 days, and then began to decrease, suggestive of an immune response against the labelled tumour cells and therefore rejection (figure 3.2.12a, figure 3.2.12b). Tumour cells were recovered from the jaw at day 35 of bone metastatic growth and cultured *in vitro* as these cells appeared to be more persistent *in vivo*. The injection and recovery process was repeated once more. The cells recovered from the second round

of bone metastasis were not rejected *in vivo* with almost all mice (9/10 mice) developing bone metastasis at a similar rate to unlabelled, parental MetBo2 cells (figure 3.2.12c, figure 3.2.12d). It should be noted that this selection process may have selected for a more aggressive subclone of MetBo2 cells, which must be considered when using this mCherry⁺ MetBo2 cell line in future experiments, particularly when comparing to any previous experiments using unlabelled MetBo2 cells.

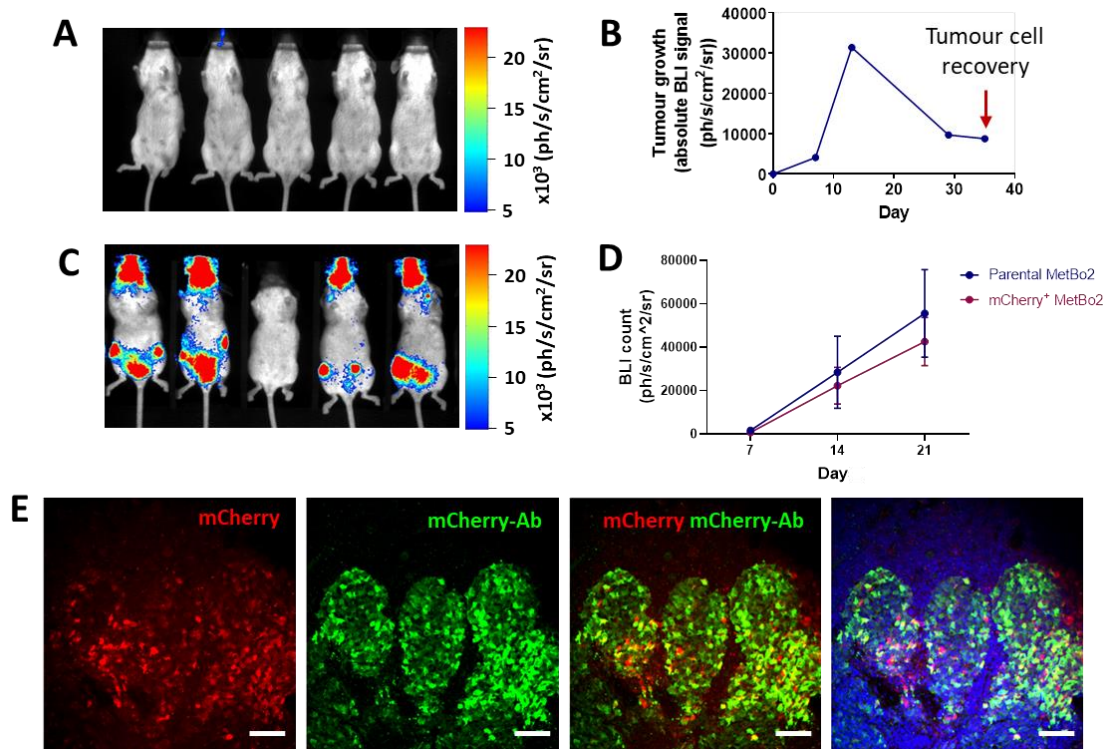


Figure 3.2.12: Selection of non-rejected mCherry⁺ MetBo2 cells *in vivo*. **A)** Bioluminescence image of FVB mice after intracardiac injection of mCherry⁺ cells recovered from subcutaneous tumours (n=5). **B)** Quantification of BLI signal of stable jaw metastasis from which mCherry⁺ MetBo2 cells were recovered (n=1). **C)** Representative bioluminescence image of FVB mice after two rounds of selection from bone metastasis. **D)** Quantification of BLI signal of bone metastasis in the femur of mice injected with parental MetBo2 cells or mCherry⁺ MetBo2 cell line (n=8-10). **E)** Immunofluorescence images of bone metastasis within the femur established from mCherry⁺ MetBo2 cells labelled with endogenous mCherry, mCherry-antibody, endogenous mCherry + mCherry-antibody and merged with DAPI (left to right). Scale bar: 100 μ m. Image shown is representative of 4 tumours collected from 4 separate mice.

The mCherry⁺ bone metastasis could also clearly be visualised using immunofluorescence imaging. Bones containing late-stage metastasis established from intracardiac injection of the mCherry⁺ MetBo2 cell line were collected and imaged as a

whole mount. The mCherry⁺ cells can be detected with a clear boundary between tumour region and normal marrow. An anti-mCherry antibody was used to overcome issues with mCherry^{low} cells in the metastasis, which are more difficult to detect (figure 3.2.12d). This subclone of the mCherry⁺ MetBo2 cells was used as the stable labelled MetBo2 cell line in later experiments.

3.2.5 Development of an intravital imaging platform

Cancer cell seeding and colonisation, particularly the tumour cell-niche interaction, is a dynamic process¹⁸⁸ and therefore real-time imaging is required to obtain an accurate insight into tumour-stromal cell interactions. To achieve this, we developed an intravital imaging platform for both the femur and calvaria.

The femur window was based on those used in previous studies with adjustments made to improve the overall design and to make compatible with our microscopes and imaging platform^{175,189}. The windows were designed using CAD-software (figure 3.2.13), and prototypes were made in plastic using three dimensional printing to test the window and imaging procedure on cadavers. This permitted adjustments to be made before the final version was generated in titanium (figure 3.2.14). The final window consists of a stabilising bar that is placed under the femur onto which the window is attached to using nuts to secure in place. The window can also be sutured to the mouse with suture holes on either side of the window. The skin-window interface and the area surrounding the bone in the window are then filled with dental cement. This provides additional adhesion to secure the window in place and creates a seal to prevent infection and leakage of fluid during imaging. The femur is then thinned using a bone drill until a thin layer of cortical bone remains so the marrow can be imaged. The window is sealed using a sterile cover slip secured with a circlip.

The calvaria window was initially designed as a rectangular window with a lock-and-key system to secure the window to the imaging platform (figure 3.2.15). However, this was unsuitable as it did not align to the curvature of the skull making it unstable. An alternative, circular window was therefore generated (figure 3.2.16). The imaging implant consists of a round window with a protruding slit on either side that secures the window in place on the imaging platform. This consists of a titanium plate with two protruding rods that attaches to the calvaria window via the protruding slits. This not only secures the window in a horizontal position, but ensures the sample remains constant and static. The window also has a lid that seals the exposed bone from the

environment during housing. A novel intravital imaging set up had to be developed on the microscope, including the arrangement of anaesthesia equipment and organisation of monitoring equipment.

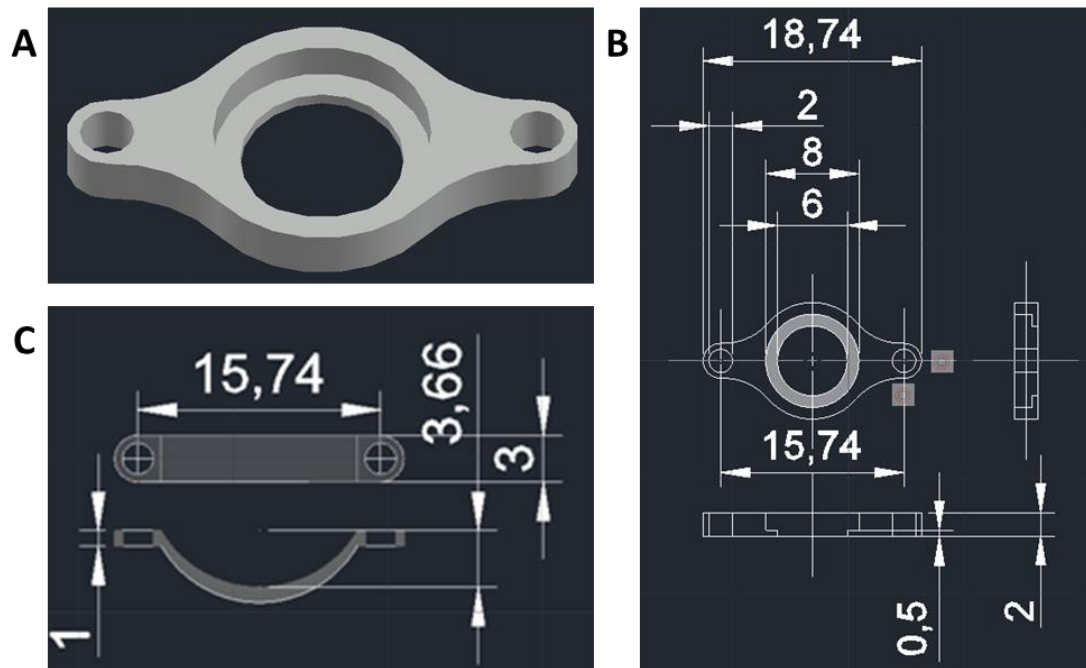


Figure 3.2.13: Femur window design. A) CAD-drawing of initial femur window. B) Dimensions of femur window (mm). C) Dimensions of stabilising bar (mm).

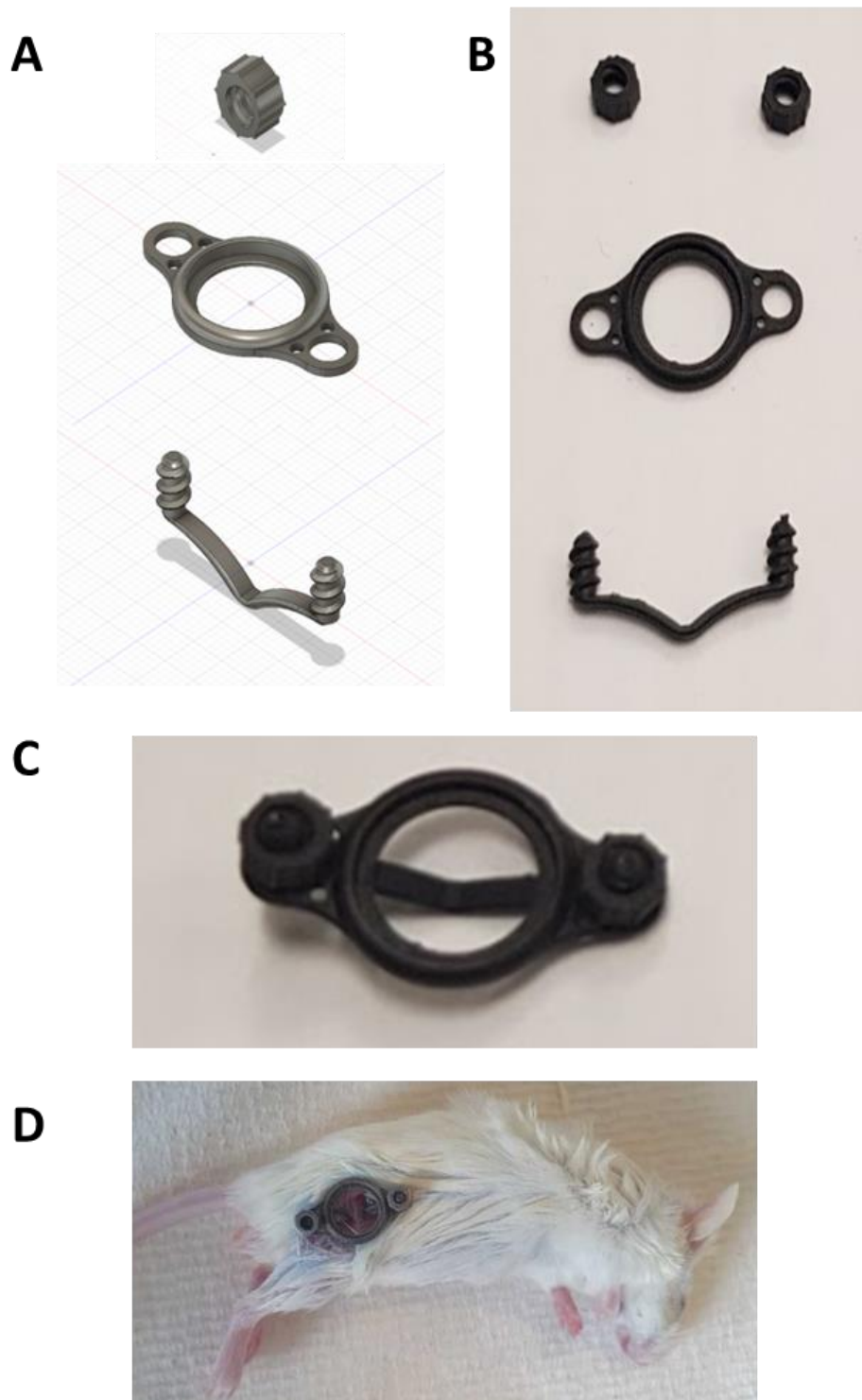


Figure 3.2.14: Finalised femur window. A) CAD-drawing of femur window components. **B)** 3D-printed prototype of femur window components. **C)** Assembled prototype of femur window. **D)** Surgical implantation of femur window on the femur of in situ for imaging.

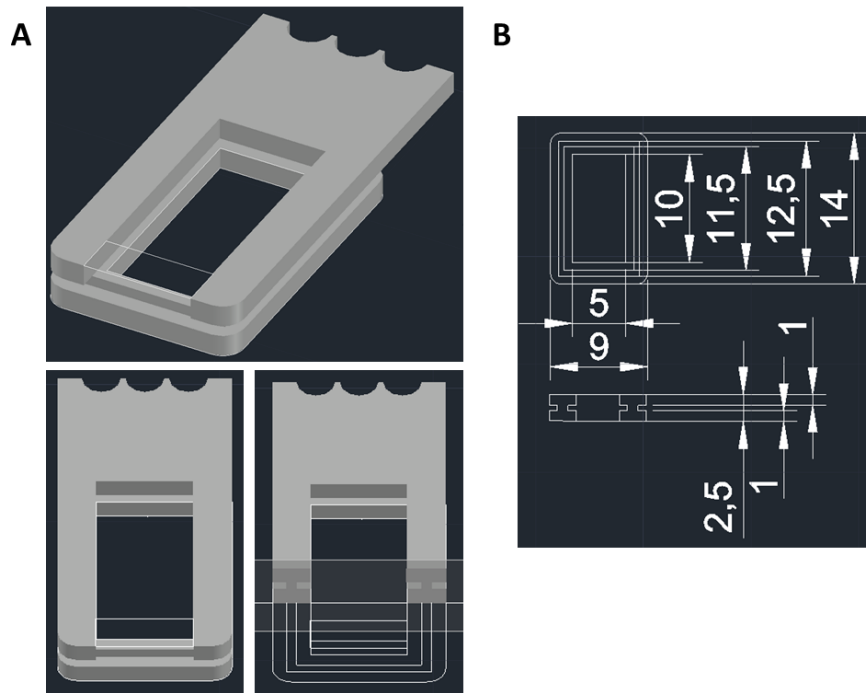


Figure 3.2.15: Initial proposed calvaria window design. A) CAD-drawing of initial rectangular-based design of calvaria window with a lock-and-key system to hold to the platform. **B)** Dimensions of rectangular window.

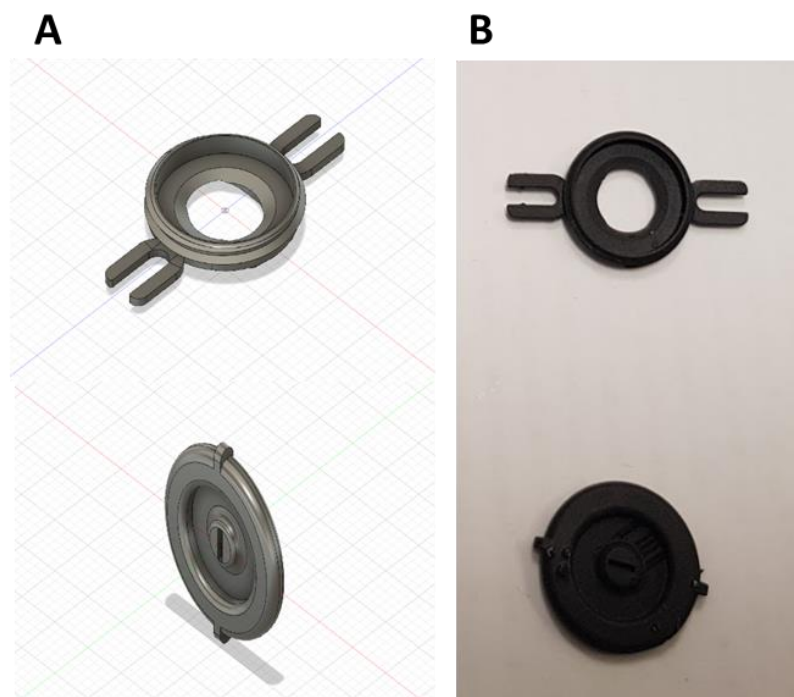


Figure 3.2.16: Finalised calvaria window and lid. A) CAD-drawing of calvaria window and lid. **B)** 3D-printed prototype of calvaria window and lid. **C)** Surgical implantation of femur window on the femur of in situ for imaging.

3.2.6 Three dimensional imaging of the bone using tissue clearance

To obtain a three dimensional image of the whole bone, the tissue must first be cleared to permit microscopic imaging. The tissue clearance process was first optimised using several clearing reagents available. Benzyl alcohol;benzyl benzoate (BA:BB) was initially used, but did not completely clear the tissue obscuring the image. Whilst substitution of benzyl alcohol with polyethylene glycol (PEG) creating BB:PEG¹⁷³, was subsequently utilised to overcome these issues, the penetrance into the bone remained a problem during imaging (data not shown). We next utilised RapiClear1.52®, which obtained complete clearance overnight (figure 3.2.17a). The bones were also shaved using a cryostat to thin the bone and expose the marrow. For imaging, the bones were placed in a chamber consisting of the bone mounted in RapiClear1.52® between two glass coverslips edged with an iSpacer® (figure 3.2.17b). To test the clearance efficiency, imaging depth and the toxicity of the RapiClear1.52® on the degradation of fluorescent proteins, stained bones were imaged at the macroscopic level using optical projection tomography (OPT) using autofluorescence to identify the overall structure (figure 3.2.18a) and at the microscopic level using confocal microscopy (figure 3.2.18b). There were no issues with imaging and a strong fluorescence signal was detected using confocal microscopy.

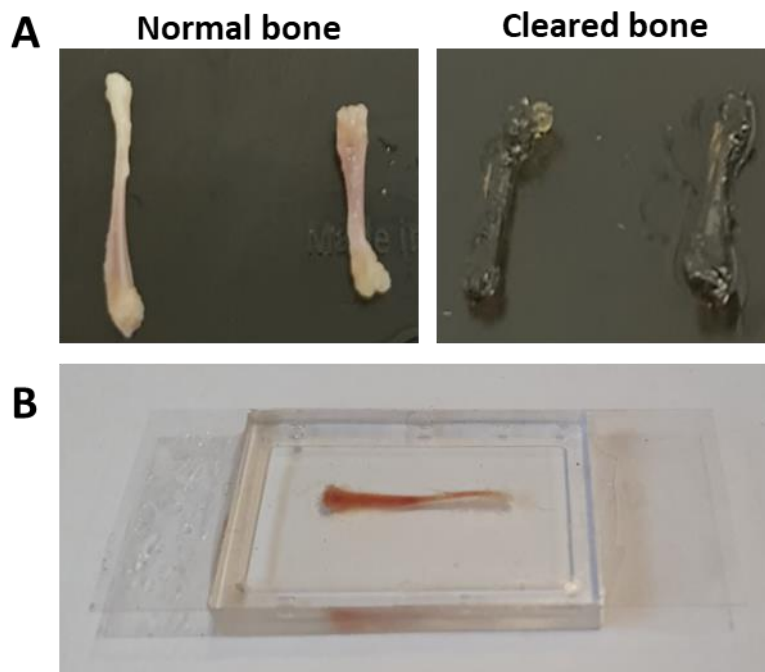


Figure 3.2.17: Tissue clearance of mouse bones with RapiClear1.52. A) Mouse bones before (left) and after (right) tissue clearance with RapiClear1.52. **B)** Chamber for imaging of cleared bones consisting of the bone mounted in RapiClear1.52® between 2 coverslips edged with an iSpacer®.

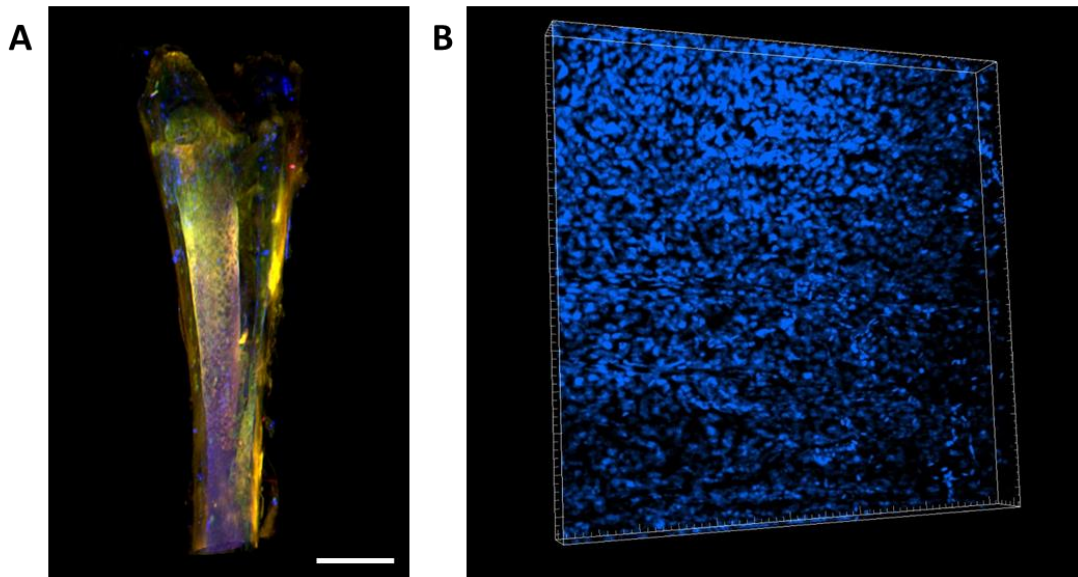


Figure 3.2.18: Cleared bones can be imaged at both the macroscopic and microscopic level. **A)** Optical Projection Tomography image of cleared bone. Scale bar: 1000 μ m. **B)** Whole mount immunofluorescence image of cleared bone marrow stained with DAPI. Scale bar: 70 μ m. (n=1, only one image acquired for each technique to confirm technique could be successfully utilised for experimental samples).

We also wished to confirm that fluorescence was maintained after clearance in RapiClear1.52[®]. Previous data in the lab demonstrates that CD204 and IL4R-expressing macrophages play a pivotal role in breast cancer bone metastasis. These macrophages were therefore imaged using in cleared bones to determine the three dimensional positioning and location of the populations in metastasis (figure 3.2.19a, figure 3.2.19b).

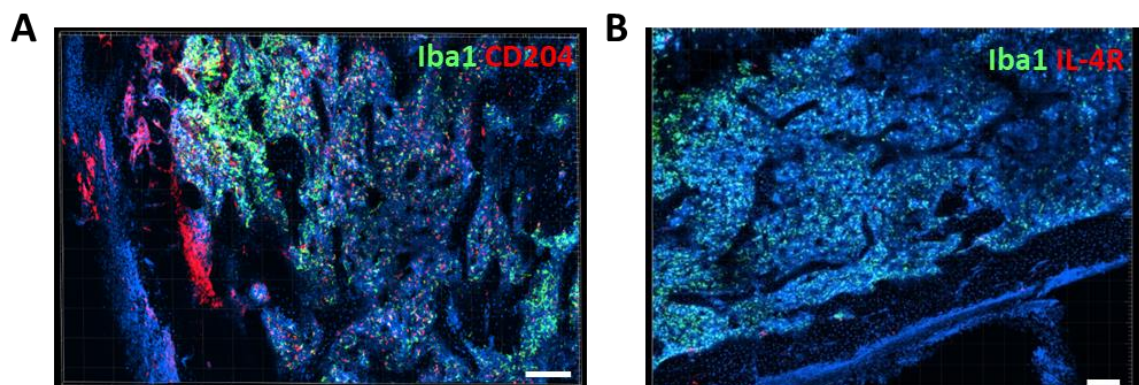


Figure 3.2.19: Macrophage populations in cleared bone. Immunofluorescence 3D images of mouse femurs stained for **A)** Iba1 + CD204 and **B)** Iba1 and IL-4R, and DAPI. Scale bar:150 μ m. Representative images of 3 separate bones collected from 3 separate animals are shown for each marker combination.

3.3 Discussion

3.3.1 Role of macrophages in breast cancer bone seeding

Tumour cell seeding to the bone is a key, rate-limiting step in the formation of bone metastasis. We have demonstrated that macrophages play a significant role in breast cancer cell seeding to the bone. Macrophage depletion during the seeding period both reduces the number of tumour cells in the bone at the seeding stage, and significantly delays bone metastatic growth resulting in enhanced survival. This is similar to the effect of macrophage depletion observed in breast cancer lung metastasis. Qian *et al.*¹⁴⁴ who demonstrated that macrophage depletion achieved using three independent mechanisms, including liposome clodronate, *Csf1r^{op/op}* mice and CD11b-DTR mice, resulted in reduced metastatic seeding and growth of breast cancer lung metastasis¹⁴⁴.

It should be noted that the single dose of liposome clodronate resulted in a 25% reduction in macrophages 3 days after injection and that it is difficult to determine the kinetics of the macrophage depletion. Additional injections may also be required to maintain the macrophage depletion throughout later stages of the seeding. Nevertheless, despite only 25% reduction in macrophages, it results in a significant reduction in tumour cells at the seeding stage and delays tumour growth. The reduced number of MetBo2 cells within the macrophage depleted bones was not due to a reduced immune rejection that would eliminate the tumour cells as the MetBo2 cells are synergetic with the FVB/N and share the same background, thus would not elicit an immune response.

The effect of macrophage depletion on seeding requires confirmation in other macrophage depletion models including CD11b-iDTR mice, and is particularly important as liposome clodronate will also cause some depletion of the osteoclasts, which have been previously implicated in early stage bone metastasis¹⁹⁰. As liposome clodronate depletes all macrophages, the origin of macrophages involved in seeding can also be determined using CD169-DTR and CCR2-knockdown mice that will deplete resident or recruited macrophages, respectively. The subset of macrophages involved in the seeding process should also be investigated using flow cytometry or fluorescence imaging by comparing the macrophage phenotype in bones after tumour cell injection with PBS-injected controls. The effect of macrophage depletion on tumour cell seeding to the bone should also be confirmed in spontaneous metastasis models of breast cancer. Whilst the experimental model used enables investigation into

the role of macrophages in tumour cell seeding independent from the primary tumour, it is also important to incorporate factors secreted from the primary tumours in the mechanism, as these modulate the pre-metastatic niche at distant sites^{187,191}.

3.3.1.1 Recruitment and activation of macrophages during seeding

The recruitment of myeloid cells to the pre-metastatic niche precedes the development of metastasis in several models and it is now recognised that bone marrow-derived cells significantly contribute to the formation of the metastasis^{192–196}. The ability of tumour cells to seed to the metastatic niche is dependent on this accumulation of myeloid cells in many cancer models, which is often mediated by interactions with factors secreted from the primary tumour, including CCL2-CCR2 and VEGFR1. In an experimental model of Met1-derived breast cancer lung metastasis, which enables investigation into the metastatic macrophages independent of the primary TME, a distinct population of CD11b⁺, VEGFR2⁺ CCR2⁺ F4/80⁺ CSF1R⁺ Gr1⁻ macrophages was recruited to the site of extravasating tumour cells. These macrophages display a pro-tumorigenic phenotype that promotes extravasation, seeding and survival, and macrophage depletion resulted in significantly reduced lung metastasis as a result of reduced tumour cell seeding. The administration of liposome clodronate prior to intravenous injection of tumour cells reduced the number of tumour cells surviving in the lung during the first 4 days of seeding, measured using RT-PCR of the tumour cell-associated genes. The results were also confirmed using CD11b-iDTR mice and also in an experimental model of human breast cancer MDA-MB-231 cells. Characterisation of the macrophages revealed that they strongly express CX3CR1, CCR2 and VEGFR1¹⁴⁴. Deficiency of the latter using a mouse model of macrophage-specific VEGFR1 deficiency as well as VEGFR1 inhibitory antibodies, resulted in reduced metastatic nodules in the lung, owing to reduced seeding as well as reduced growth¹⁸⁶.

VEGFR1 has also been implicated in the formation of the liver and lung pre-metastatic niche of Lewis lung carcinoma. The Lewis lung carcinoma cells induced a systemic signalling response that promoted fibronectin expression at the niche in the liver and lung. Subsequently VEGFR1⁺ bone marrow-derived cells expressing the fibronectin receptor, known as VLA4, are attracted to this fibronectin-rich region, and promote chemoattraction and adherence thus colonisation of CTCs. Albeit, the exact type of bone marrow-derived cell was not identified, but was of haematopoietic origin. Recruitment of bone marrow-derived haematopoietic progenitor cells preceded the arrival of tumour cells, and was VEGFR1-dependent as inhibition of VEGFR1 using

neutralising antibodies prevented accumulation of bone marrow derived cells in the lung and subsequent metastasis. Ablation of the tumour-derived signalling also reduced the bone marrow cell accumulation and therefore metastasis. Moreover, similar results were observed in metastasis of B16 melanoma¹⁹⁷. The role of VEGFR1 in recruitment of macrophages to the metastatic lung of Lewis lung carcinoma and B16 melanoma has been further confirmed by others, where VEGFR1 inhibition significantly reduced metastasis¹⁸⁰. In addition, VEGFR1 is also important for the colonisation and survival of acute lymphoblastic leukaemia cells in the bone marrow niche. Inhibition of VEGFR1 using neutralising antibodies increases cancer cell apoptosis and prolonged survival of the mice¹⁹⁸.

CD11b⁺, CD68⁺, F4/80⁺ and CXC3CR1⁺ macrophages are also recruited to the pre-metastatic niche of melanoma lung metastasis in a manner dependent on tissue factor from the primary tumour¹⁹⁹. Lewis lung carcinoma and B16 melanoma cells also secrete TNF α , VEGFA and TGF β , which induce production of S100A8 and S100A9 from both the endothelial cells and macrophages within the lung. This results in recruitment of mac1⁺ myeloid cells to the pre-metastatic lung of B16 melanoma and Lewis lung carcinoma models. These macrophages subsequently attract tumour cells to the lungs, which could be prevented using S100A8 or S100A9 inhibitory antibodies²⁰⁰. S100A9 may further enhance retention of myeloid cells at the pre-metastatic niche by activating β_2 integrins, such as CD11b, which enhances adhesion to fibronectin²⁰¹. It is also associated with vascular permeability and therefore may mediate extravasation of the tumour cells²⁰². Other factors involved in recruitment of macrophages to the pre-metastatic niche include CCR2-CCL2 interactions between CCR2⁺ Gr1⁺ inflammatory monocytes and CCL2-expressing tumour cells, which promotes recruitment of macrophages to the lung prior to the arrival of tumour cells. Here, the CCR2-monocytes differentiate into MAMs that support metastatic seeding by promoting extravasation of the tumour cells¹⁴⁵.

Tissue resident macrophages are also activated at the pre-metastatic site by primary-tumour derived factors, including exosomes, and promote localisation to specific organs^{181,203}. Interestingly, exosomes also display organotropic effects during the preparation of the pre-metastatic niche by preferentially interacting with the cells at the predicted secondary site. For example, exosomal integrins $\alpha_6\beta_1$ and $\alpha_6\beta_4$ secreted from lung-trophic tumour cells associate with lung metastasis, whereas exosome integrin $\alpha_v\beta_5$ from liver-trophic tumour cells is involved in the pre-metastatic niche in liver

metastasis. At each of the sites, the relevant exosomes localise with and are engulfed by macrophages in fibronectin-rich regions to promote metastasis. Targeting the integrins reduces lung and liver metastasis. This highlights the differential mechanisms between metastatic sites and the requirement to investigate each separately²⁰⁴.

3.3.1.2 Mechanisms mediating seeding

3.3.1.2.1 Macrophage-mediated niche

Macrophages accumulated at the premetastatic niche support tumour cell seeding and colonisation through several mechanisms. Macrophages directly secrete factors that act as chemoattractants to induce tumour cell recruitment and extravasation. MAMs derived from CCR2⁺ Gr1⁺ inflammatory monocytes, described above, promote extravasation in a VEGF-dependent manner. Specific ablation of VEGFA in the myeloid cells using a transgenic mouse model where inducible Cre is driven by the *Csf1r* promoter is crossed with *Vegfa*^{flox/flox} mice, significantly reduces extravasation resulting in attenuated number of metastatic nodules in the lungs. This is further supported using *in vitro* migration assays where tumour cell transmigration across an endothelial layer towards bone marrow-derived macrophages was completely attenuated using VEGFA-depleted macrophages. VEGFA-depleted mice were also unable to enhance permeability of the endothelial cell layer¹⁴⁵. In contrast, data in this project suggests that myeloid-derived VEGFA does not contribute significantly to breast cancer cell seeding in the bone. Using the same transgenic mouse as Qian *et al.*¹⁴⁵ we demonstrate that myeloid-specific VEGFA depletion during seeding has no significant effect on the tumour cell number during early stage seeding or late tumour growth. However, it should be noted that whilst efficiency of VEGFA-depletion in this model is high in lung macrophages, this needs to be confirmed in bone macrophages using qPCR of flow-sorted immune cells and is ongoing. Nevertheless, the VEGFA-dependent seeding mechanisms observed in previous studies may be specific to the lung with alternative mechanisms being utilised by the breast cancer cells in the bone. This suggests that distinct populations of macrophages may exist in the lung and the bone, and exhibit site-specific effects. The model used will deplete VEGFA in all CSF1-expressing macrophages, including resident and recruited populations, and therefore differences between resident alveolar macrophages in the lung and bone marrow macrophages may contribute to the differences observed²⁰⁵. Differences between the metastatic niche in the lung and bone have also been previously described. For example, Dickkopf-related protein 1 (DKK1) inhibits macrophage and neutrophil

recruitment and therefore prevents metastasis in the lung, whereas DKK1 promotes bone metastasis by attenuating the Wnt factors that usually inhibit macrophage to osteoclastogenesis²⁰⁶. Another study also demonstrated differential roles of the bone and lung vasculature during seeding. Endothelial-selectin (E-selection) in the vascular niche facilitates extravasation and colonisation to the bone by directly interacting with the tumour cells and inducing Wnt signalling to promote survival. This was attenuated in E-selectin-deficient mice resulting in delayed bone metastatic progression. This role of E-selection was also specific to the bone with colonisation to both lung and liver metastasis being unaffected by E-selectin depletion²⁰⁷. The endosteal and perivascular HSC niches in the bone also provide a unique environment for tumour colonisation that does not exist in the lung and therefore lung metastatic cells must utilise alternative niches. Macrophages within the bone may therefore exert site-specific effects on the HSC niche, and will be discussed later. Moreover, breast cancer cells exhibit distinct site-specific phenotypes that mediate tropism towards different metastatic sites^{95,208}. These may therefore also establish different interactions with the macrophage populations during colonisation. To determine the macrophage-mediated mechanisms in the bone, further characterisation of macrophages in control and metastatic bones during seeding should be performed using techniques such as flow cytometry and RNA-sequencing. Potential mechanisms will then be confirmed functionally using siRNA knockdown in the tumour cells and transgenic mice with specific depletion of macrophage-expressed molecules. Alternative macrophage-mediated mechanisms to attract and retain tumour cells at the metastatic niche have been identified in other cancer models and secondary sites, and may offer insight into potential mechanisms.

In a model of breast and lung cancer brain metastasis, exosomes containing CEMIP released from tumour cells are uptaken by microglial and endothelial cells resulting in upregulation of pro-inflammatory cytokines such as cyclooxygenases (COX) and TNF in the microglia. These promote brain vascular modelling, extravasation and subsequent metastasis. CEMIP depletion impaired bone metastasis by disrupting the invasion process and tumour-vasculature interactions. Interestingly, this process is specific to the metastatic site with CEMIP being elevated in exosomes derived from brain metastasis, but not the lung or bone, and only predicted brain metastasis progression and patient survival²⁰⁹. During metastasis of melanoma to the brain, soluble factors are released from the tumour cells, which activate the inflammatory-like response within the brain. Subsequently, monocyte-derived CXCL10 is released and

acts as a chemoattractant to induce CXCR3-expressing melanoma cell migration and extravasation. Targeting of CXCR3 prevents the formation of brain metastasis²¹⁰.

Macrophages also establish direction interactions with the tumour cells to facilitate adhesion to the metastatic niche. For example, VCAM1-integrin α_4 interactions mediate adhesion of VCAM1-expressing tumour cells to α_4 -expressing MAMs in the metastatic lung of breast cancer. Whilst VCAM1 knockdown in MDA-231-LM2-4175 breast cancer cells significantly reduced lung metastasis, primary tumour growth is not affected²¹¹. A similar VCAM1-mediated interaction was also observed during seeding and colonisation of melanoma cells in the lung. Portions of cytoplasm from cancer cells, known as cytoplasts, arrested and remained in the vasculature. This preceded the arrival of neutrophils, monocytes and non-alveolar macrophages, which engulfed the cytoplasts and subsequently expressed adhesion molecules, including VCAM1. This created a pre-metastatic niche preceding the arrival of tumour cells and promotes extravasation into the lung²¹²

A critical component of the pre-metastatic niche is ECM proteins, such as tenascin C, periostin and fibronectin, that provide anchorage through cell surface receptors such as integrins and CD44^{213,214}. Macrophages also mediate the composition and stiffness of the ECM at the pre-metastatic niche through the secretion of ECM components as well as remodelling enzymes. Lysyl oxidase (LOX) derived from hypoxic breast cancer cells induces collagen IV crosslinking in the basement membrane of the lung. This induces recruitment of CD11b+ myeloid cells, which cleave the collagen via the secretion of MMP2 and subsequently promotes adhesion of breast cancer cells to the niche. Together, this creates a pre-metastatic niche that promotes tumour cell recruitment to the lungs. CD11b+ cells are found to be colocalised with LOX in patient biopsies, further supporting a role¹⁷⁹. Interestingly, LOX also mediates the formation of pre-metastatic niche in the bone²¹⁵. Macrophage-derived MMP9 is also induced by the Lewis lung carcinoma and B16 melanoma in a VEGFR1-dependent manner prior to lung metastasis. This promotes the formation of metastasis, whereas MMP9 blockade resulted in significantly less metastasis¹⁸⁰. Metastasis associated macrophages also secrete granulin that activates hepatic stellate cells into myofibroblasts, which subsequently secrete periostin and collagen creating a fibrotic niche for liver metastasis of pancreatic cancer²¹⁶. In a model of liver metastasis of pancreatic cancer, exosomes from the primary cancer cells also activate macrophages into a pro-fibrotic phenotype at the secondary site priming the environment for the tumour cell arrival. Initially,

cancer cell-derived exosomes containing MIF are taken up by liver-resident macrophages, Kupffer cells, which results in TGF β secretion and upregulation of fibronectin by hepatic stellate cells. Subsequently, this pro-inflammatory, fibrotic-like environment promotes recruitment of bone marrow-derived macrophages to the liver, which promote tumour cell colonisation. Macrophage depletion using the CD11b-DTR mouse model reduced metastasis after injection of the tumour cells into exosome-educated mice¹⁸¹. A similar macrophage-dependent niche is observed in an orthotopic model colon cancer where total macrophage depletion or Kupffer cell depletion attenuated the development of liver or peritoneal metastasis. Furthermore, the number of liver metastasis correlated with the Kupffer cell density suggestive that they promote seeding²¹⁷.

3.3.1.2.2 *Modulation of the HSC niches by macrophages*

Many of the studies investigating the macrophage-mediated regulation of the pre-metastatic niche have been performed in either lung or liver metastasis with the role of macrophages in tumour cell in the bone yet to be determined. Nevertheless, both the endosteal and perivascular HSC niches have been identified as pivotal niches for bone metastatic tumour cells^{59,88,103,182}. We also show that the MetBo2 cells seed to the osteoblast-rich region with the majority of tumour cells seeding to the region below the epiphysis plate. This supports previous studies demonstrating that the niche is also located within the trabecular region or osteoblast-rich of the bone in breast cancer¹⁸² and prostate cancer bone metastasis²¹⁸. These studies demonstrate the functional role of the HSC endosteal niche indirectly by using CXCR4 antagonists, AMD3100, to mobilise the HSCs from the niche allowing the cancer cells to occupy the space instead. The CXCR4 inhibition also affects other cells within the bone and there is a lack of evidence demonstrating the effects of the direct depletion of osteoblasts only. Therefore, using the SP7-GFP-Cre-DTR mouse model, we can investigate the effects of osteoblast depletion on tumour cell seeding, and hypothesise that depletion of the osteoblasts will significantly reduce tumour cell seeding to the bone and delay tumour growth.

During bone colonisation, melanoma cells also interact with the perivascular niche composed of MSCs and pericytes that facilitate extravasation and seeding. CD146-deficient cells or PDGF-BB deficient mice fail to develop bone metastasis after intra-arterial injection of melanoma cells⁹¹. Future experiments will therefore also investigate the role of MSCs and pericytes in breast cancer bone metastasis using the seeding

model in Nestin-iDTR, NG2-DTR and PDGFR β -DTR mice to determine the mesenchymal population that may be responsible.

Tumour cells seed to the endosteal and perivascular HSC niches by forming adhesions with the osteoblasts or MSCs/pericytes, respectively. Wang *et al.* demonstrated that heterotypic adheren junctions consisting of cancer-derived E-cadherin and osteoblast-derived N-cadherin form key interactions at the osteogenic niche. Inhibition of the interactions reduces the number of tumour cells seeding and delays tumour growth⁸⁸. Gap junctions, composed of connexin-43, also form between breast cancer cells and osteogenic cells, which permit the transfer of calcium to the bone metastatic cells to promote their survival¹⁰³. Other stromal-derived chemoattractants and adhesion molecules have also been implicated in other types of bone metastasis, including annexin II and CXCL12 in prostate cancer^{98,219,220}. Within the perivascular niche, melanoma cells also seed to the pericytes in a CXCL12-dependent manner²²¹. This is further supported by Price *et al.*²²² who demonstrate the breast cancer cells occupy CXCL12-CXCR4-rich areas during early bone metastasis. Inhibitors of the CXCL12-CXCR4 signalling pathway prevented breast cancer extravasation, entry to the bone marrow, and anchorage to the niche²²².

It has been identified that macrophages play a pivotal role in maintaining the MSCs and osteoblasts and thus retention of the HSCs within both the perivascular and endosteal niche in normal bone, respectively^{71,83,184}. Due to the notable similarities between the retention of HSCs and tumour cells in the niche as previously discussed in chapter 1, we hypothesise that macrophages may exert similar effects during tumour cell colonisation of the HSC niche. Furthermore, macrophages may promote or reinforce the interactions and adhesions between the tumour cells and niche cells, such as cadherin, annexin and CXCL12 described above. For example, it has previously been shown that E-cadherin expression is regulated by CD206⁺ macrophages²²³. Future experiments will therefore involve investigating the effect of macrophage depletion on seeding to the macrophages and the endosteal or perivascular niche. To determine the location of tumour cells in relation to macrophages, osteoblasts and MSCs during seeding, we have developed several imaging techniques including intravital imaging of the femur and three dimensional imaging of cleared tissues. Together, this will allow both accurate and holistic measurements as well as imaging in real time throughout the seeding process. Using FMacGreen, SP7-GFP and Nestin-GFP or NG2-dsred mice, labelled tumour cells can be visualised microscopically via the femur window. This will

permit imaging and monitoring of the movement of tumour cells in the circulation (labelled with Dextran), the extravasation process, and the seeding in relation to fluorescently labelled macrophages, osteoblasts or MSCs/pericytes. Bone samples will also be collected at specific/regular time points throughout the seeding period for tissue clearance and three dimensional confocal imaging. This will allow visualisation and quantification of the total number of surviving tumour cells in the bone as well as the distance between the tumour cells and stromal cells. The effect of macrophage depletion on seeding and extravasation can also be studied by administering liposome clodronate prior to injection of labelled tumour cells. We hypothesise that that macrophage depletion will reduce the number of surviving tumour cells in the bone similar to that observed using the BLI signal, and increase the tumour-stromal distance. Furthermore, we will use super-resolution imaging of the cleared tissues to investigate the effect of macrophage depletion on the formation of the adhesion molecules between the tumour and stromal cells. If confirmed *in vivo*, functional assays including migration assays will be performed to test molecular mechanisms using neutralising antibodies and molecular knockdown.

Macrophages also regulate osteogenic differentiation of MSCs to maintain the osteoblasts within the endosteal niche, and therefore may increase the capacity for tumour cell seeding by expanding the niche⁴² (figure 3.3.1). A similar induction of MSC

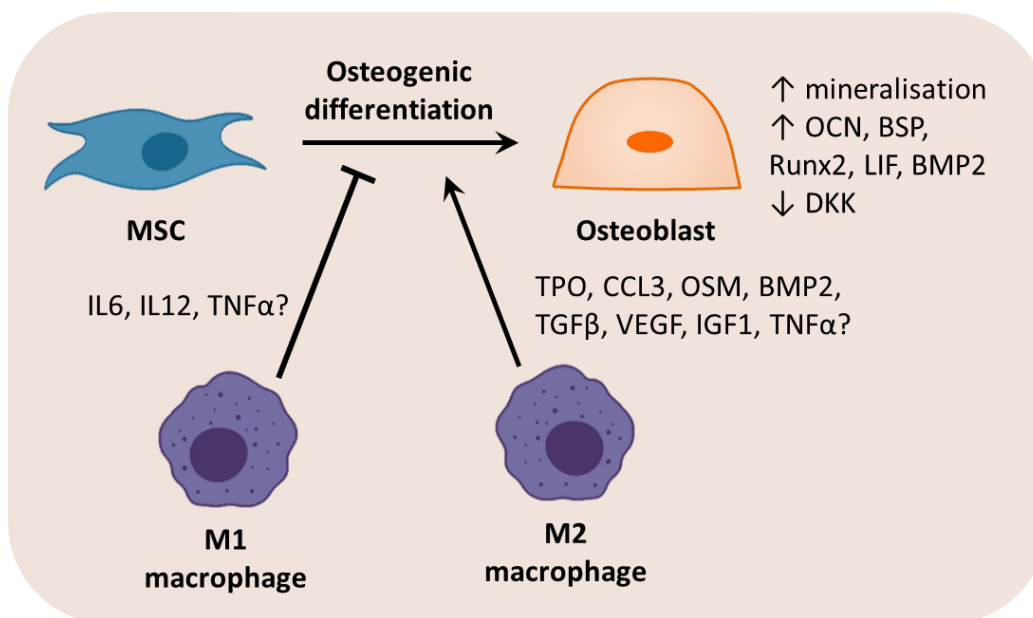


Figure 3.3.1: Macrophage regulation of osteogenic differentiation. M1-like macrophages predominantly inhibit the osteogenic differentiation, whereas it is promoted by M2-like macrophages via the secretion of soluble factors.

to osteoblast differentiation has been observed in other cancer models. Myeloid cells promote aberrant MSC to osteoblast differentiation in a model of leukaemia, which is driven by synergistic interactions of thrombopoietin and CCL3 as well as other unidentified interactions. This results in an overproduction of functionally abnormal osteoblasts that exhibit altered TGF β , notch and inflammatory signalling. These osteoblasts accumulate in the bone marrow and support the retention of the leukaemic cells. Although the function of these myeloid cells were similar to the role of monocytes and macrophages that regulate bone marrow niches, the identify of myeloid cells were not characterised beyond the expression of Mac1²²⁴. Similarly, in prostate cancer bone metastasis, haematopoietic progenitor cells were capable of inducing osteoblast differentiation, which was partly dependent on BMP2. Notably, haematopoietic progenitor cells isolated from non-metastatic bones were expressed comparably low levels of BMP2 and were unable to induce these changes, suggestive of a tumour-specific effect in the haematopoietic progenitor cells. Furthermore, these effects were specific to osteoblastic bone metastasis, whilst in osteolytic models HSCs drive macrophage to osteoclast differentiation and subsequent bone metastasis via IL6 signalling²²⁵. The BMP2-oncostatin M (OSM) signalling pathway is also a key pathway involved in macrophage-mediated osteoblast differentiation in non-tumour bone. Zhang *et al.* initially demonstrated that oncostatin M and BMP2 were increased in macrophage-treated osteoblasts, although this was not tested functionally. Others have, however, confirmed a functional role of oncostatin M and BMP2 using siRNA knockdown and neutralising antibodies^{46–49,226}. The overlap in the role of BMP2 in osteoblast differentiation in normal and tumour-bearing bones, also indicates that macrophage-derived factors implicated in osteogenic differentiation of MSCs identified in non-tumour bone may offer insight into the molecular mechanisms involved in metastatic bones. Gong *et al.* demonstrated that M2 macrophages secreted TGF β , VEGF and IGF1 to promote differentiation, whereas M1 macrophages secreted IL6, IL12 and TNF α to inhibit differentiation⁵¹. As tumour-associated macrophages are phenotypically similar to M2 macrophages, which are involved in osteoblast differentiation of MSCs, they may play a similar role. Another study also suggested that M2 macrophage-derived TNF α was involved in promoting osteoblast differentiation, however, this may be an artefact of the lipopolysaccharide stimulation⁴². Macrophage-derived exosomes have also been shown to induce osteogenic differentiation after internalisation into the MSC. Both macrophage-derived conditioned media and exosomes isolated from conditioned media were able to induce both Runx2 and BMP2

in MSCs resulting in osteoblast differentiation. However, the contents of the exosomes responsible for the effects were not studied⁴⁸. Finally, macrophages also induce release of cytokines from MSCs that may promote colonisation. Macrophage-derived IL8, CCL2 and CCL5 promotes release of cytokine release from MSCs, including IL6, CCL5 and CXCL10²²⁷. Interestingly, these MSC-derived molecules have been implicated in bone metastasis colonisation and progression^{228–234}. We hypothesise that similar macrophage-osteoblast/MSC interactions and mechanisms may be observed in bone metastasis.

It is evident that macrophages play a role in seeding of the breast cancer cells to the bone. Further research is required to identify the mechanisms involved and whether tumour cells seed directly to the macrophages or if macrophages mediate alternative niches within the bone. The mechanisms observed in other metastasis models as well as those observed in normal bone, summarised in figure 3.3.2, may offer insight for future investigations in our model.

3.3.2 Development and optimisation of novel techniques to image the bone

3.3.2.1 Intravital imaging

Traditional microscopic imaging of biological samples provides static information from a fixed sample in 2D usually using immunohistochemistry, immunofluorescence or flow cytometry analysis of samples. This snapshot is dependent on the tissue status at the point of sacrifice. However, biological processes including metastatic seeding and colonisation are dynamic occurring in real-time and in three dimensional environments. Therefore traditional imaging of sections often omits important information such as three dimensional distances and kinetic information. In order to image in real-time, live imaging is required and, whilst this is relatively simple in *in vitro* cultures, intravital imaging is required for *in vivo* studies^{235,236}. We developed an intravital imaging platform to enable live microscopic imaging of the bone in real time. This not only allows us to image the seeding process in real time, but also long-term observation of the process in the same sample in a longitudinal manner.

Intravital imaging is defined as the imaging of live animals at the microscopic, cellular or subcellular level, usually using a window that has been inserted on the organ or area of interest. The implantable device contains a transparent area permitting the microscopic imaging²³⁶. This offers insight into many biological processes including cell

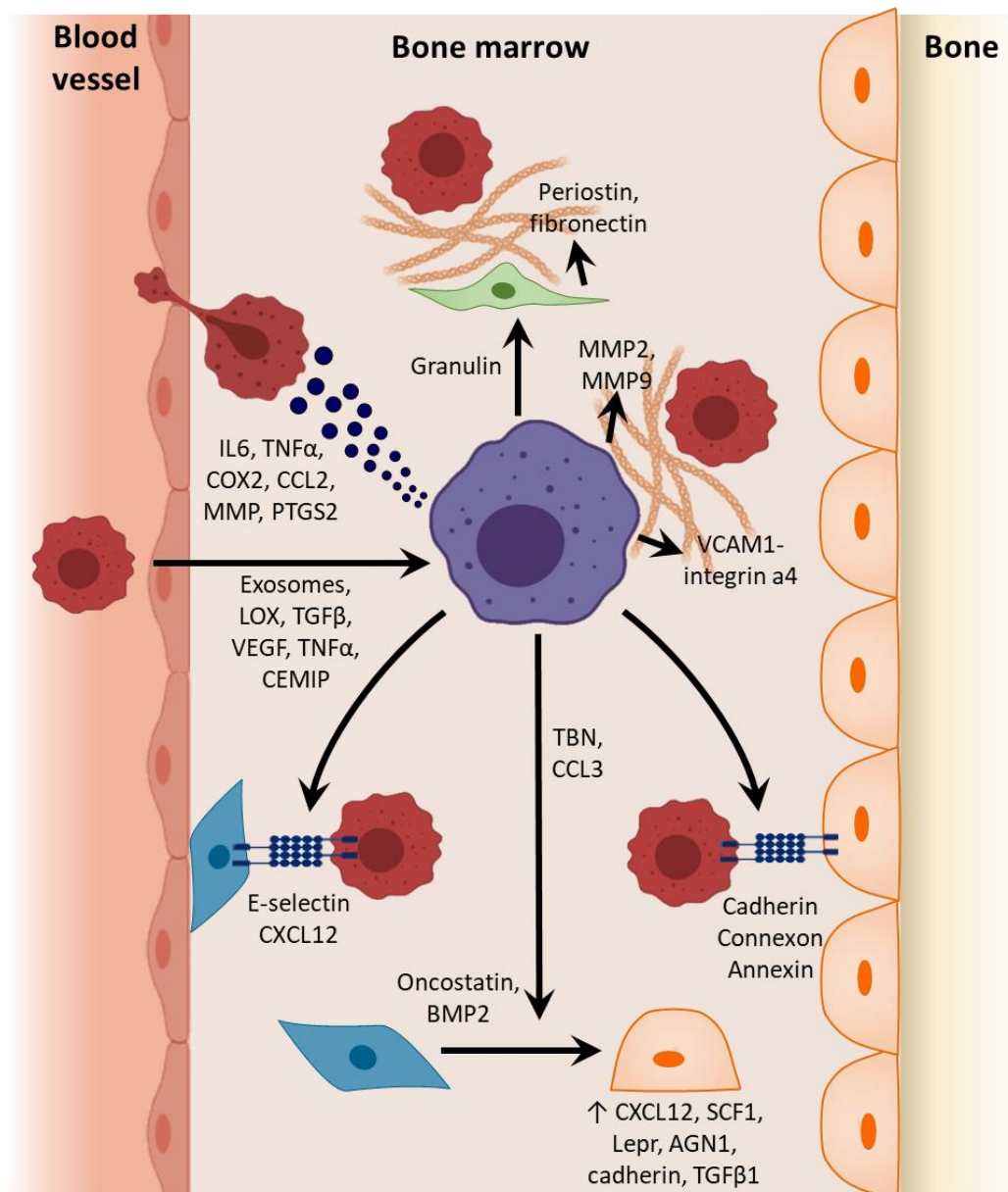


Figure 3.3.2: Macrophage regulation of tumour cell seeding. Studies within other cancer models, metastatic sites and normal tissues demonstrates that macrophages can promote seeding. Macrophages can form a niche where they secrete chemoattractants that promote extravasation and migration towards the niche. They also modulate the ECM by secreting matrix proteins, inducing matrix production in other cells, or secreting factors that modulate the existing ECM to create a favourable niche. Macrophages may also mediate other existing HSCs niches within the bone by inducing expansion of the niche or by promoting interactions between the tumour cells and niche cells.

division, cell migration, cell death, cell communications, cell fate and lineage tracing and vascular networks formation and delivery, metabolism, reviewed in^{236,237}. Basic intravital imaging was performed as early as the 1800s using bright-field microscopy of translucent tissues to image blood flow and extravasation of leukocytes from the

circulation to inflamed tissue. Intravital imaging has since hugely advanced and now involves the use of window implants to image at greater depths, higher microscopic resolution, more complex tissues and organs, and fluorescent labelling^{235,236}.

The earliest intravital windows were dorsal skin-fold window chamber models and have been used for over 70 years. This window is inserted by folding and fixing the skin between the frames of the window. It is often used to image xenografts such as tumours and remains the one of the most widely utilised windows in cancer research. It permits direct access to the tissue, however, a xenograft does not permit imaging of tissue in the native environment^{238–241}. A mammary imaging window allows imaging of the mammary gland and has been utilised to monitor both healthy and tumorigenic mammary tissues. This is similar to the skin-fold window, however, the window is inserted between the mammary gland and skin^{242–244}. Both the brain and the calvaria can be imaged with cranial windows, which are relatively easily adhered to the skull using glue. Imaging of the brain requires at least thinning of the skull²⁴⁵, or a craniotomy to remove the bone to expose the tissue²⁴⁶, whereas the bone marrow of the calvaria can be directly imaged. Whilst these sites, including the skin, breast and skull, can be easily accessed for imaging, longitudinal imaging in deeper tissues such as the abdominal organs is more inaccessible and therefore challenging. An abdominal window has now been developed to image organs such as the liver, pancreas, intestine, kidney and spleen. This involves a titanium ring that is surgically implanted into the skin and abdominal wall, held in position by a purse-string suture whilst the organ to be imaged is brought to the surface of the window²⁴⁷. However, very little has been developed to image the bone, particularly the long bones.

The femur is one of the most challenging anatomical locations to image using intravital imaging as it not only has to be surgically exposed, but movement of the leg must be maintained without causing damage. For this reason, there is also very little available tissue that the window can be adhered to. Alternative methods have been developed to image live bone, including metastasis, at the microscopic level. This includes *ex vivo* techniques such as bone-in-culture array where established bone metastases are isolated from mice and cultured *ex vivo* as whole or parts of bone, which can be manipulated and imaged²⁴⁸. Another technique was to use a dorsal skinfold window on ectopic engineered bone tissue²⁴⁹. Similarly, femurs have also been imaged ectopically after transplantation of split femurs into a dorsal skinfold chamber of a host mouse²⁵⁰. Whilst these approaches permit normal biological processes, including bone

regeneration, they do not allow *in situ* validation of processes as the normal function, structure and anatomical site of the bone is significantly disrupted. Additionally, it did not mimic the organised bone structure, particularly that of long bones. Windows to image the bone *in situ* are therefore essential.

The earliest bone chamber implant was first described by Braenmark in 1959 and was utilised to image the rabbit tibia. It consisted of a hollow titanium cylinder with a screw thread on the external surface, which could be implanted bicortically in the bone. Implantation involved drilling a hole through the bone and inserting the cylinder/screw through the hole. The cylinder contained a slit in which outgrowths of regenerating bone marrow would grow through and fill the gap into the observation area, which can subsequently be imaged from above the cylinder. This initial chamber was altered by several research groups throughout the 1970-1980s, but maintained the same basis of the design. Whilst these could be used for long-term observation for over 1 year and was used to monitor microcirculation, as the bone had to grow into the slit before imaging, a 6-9 week delay was required whilst the bone grew into the slit gap. This also meant that only new forming bone could be imaged. Furthermore, this design was only suitable for larger animals/rodents to ensure the cylinder was large enough for a suitably sized observation area. The design also meant that the bone was no longer biomechanically active, which is important in bone remodelling and homeostasis^{251,252}. A recent imaging window was designed where it was directly screwed into the femur to secure it in place. This allowed imaging of deeper regions up to 100-500µm in the central area of the marrow, which was previously inaccessible, and for up to 115 days. However, this causes fractures in the bone, which will alter both the inflammatory response and the bone remodelling during the healing process²⁵³. An alternative approach to image the marrow was to directly expose the marrow under a terminal procedure rather than insert a window. However, this allows short term tracking rather than longitudinal imaging²⁵⁴.

Whilst these methodologies permit the direct live imaging of the bone marrow within the femur, many have limitations and are unsuitable for long term imaging of bone. The first femur window that overcame many of the previous issues including preservation of the bone integrity and mechanical loading, as well as imaging of the existing bone was developed by Hansen-Algenstaedt *et al.*¹⁸⁹. This window consisted of a flat window that lay along the bone and was held in place by a bar that is placed under the bone, known as the 'sandwich' technique. The cortical bone is then partially grinded down to expose

the marrow. This technique permitted long-term optical access to the femoral bone marrow *in situ* to directly image the microcirculation of pre-existing bone¹⁸⁹. Whilst the basic design of this femoral window is optimal, the overall window was large at about 1.2cm, and had a relatively small imaging area of 4mm. Its use was therefore limited to large mice over 3-6 months old. This design was therefore further optimised by Chen *et al.* to image the dynamics of the neutrophils. Their alterations included increasing the imaging field of view and reducing the overall size of the window, making it more suitable for smaller mice. The window was also more stable and less likely to accumulate fluid or infection due to the application of dental cement between the bone and window edge, which acts like a seal to the outer environment in addition to the coverslip. Furthermore, the glass coverslip was secured by a circlip rather than permanent adhesion therefore could be replaced, also reducing infection risk and opacity from fluids¹⁷⁵. We therefore based the design of our femur window on the 'sandwich' technique by adapting the window made by Chen *et al.*

In contrast to the femur window, the calvaria requires a much less complicated and non-invasive window. The skull, particularly the frontoparietal lobe, is <100µm thick permitting the penetration of excitation light through the tissue and therefore requires minimal surgical intervention. Calvaria windows are relatively easy to implant and involves making an incision in the skin, removing the membranes over the skull and adhering the window to the bone^{255–261}. Due to these properties, the calvaria is currently the most common and popular method to image the bone marrow *in vivo*, however, the long bones are structurally, functionally and developmentally distinct from the calvaria, and therefore separate imaging is necessary.

The most challenging aspect of intravital imaging is light scattering, which limits penetration depth to 100-200µm in confocal microscopy and up to 1mm in multiphoton microscopy. Abundant noise of out-of-focus light collected from over- and under-lying tissue also creates background signal from the excited signal-of-interest resulting in high signal-to-noise ratios. Multiphoton microscopy reduces the background signal and allows increased light penetration, and therefore we initially planned our imaging to take place using multiphoton microscopy. However, due to technical issues with the microscope, we later transferred our imaging to a spinning disk confocal. Whilst we could only image at lower penetration depths (~200µm), the signal-to-noise ratio remained reasonably low. Whilst the calvaria can be imaged directly due to the thin layer of cortical bone, the mineral exterior layer of the cortical bone in the femur

prevents direct imaging of the bone marrow. In order to overcome the optical limitations and image the marrow, surgical exposure was therefore required. Grinding the cortical bone using a bone drill approximately 50µm allows imaging in the z-dimension of 200-300µm on average²⁵⁰. Although this makes imaging of the long bones more invasive and may induce a bone-healing response, it is a necessary step to image the bone marrow²⁶².

Another key technical issue with intravital imaging is the stabilisation of the mouse during the imaging to prevent artefacts as a result of movements of the mouse, including breathing. Stereotaxic frames or mounts are used to secure the mouse to the microscopic stage. However, often conventional stereotactic holders are not suitable for imaging of the calvaria as they obstruct the frontal bones. Scott *et al.*²⁶³ developed a mouse holder that consists of an arm that attaches to the imaging window via a lock-and-key mechanism. It has a base plate at the other end of the arm, which is secured into the microscope stage. Together, this not only is less distressing to the mouse during live imaging and allows normal breathing, but stabilises the mouse enough to eliminate movement artefacts due to breathing^{263,264}. We therefore adapted this lock-and-key mechanism, but instead had the attachment area on either side of the window, and attached to two vertical rods on a titanium plate. This secured the head of the mouse in the same stationary, horizontal position independent of other movements, whilst having no restrictions on breathing.

During longitudinal studies, it is important to return to the original location of the tissue being imaged. This can be challenging, particularly at the microscopic level. We will adopt several previously used techniques to aid locating the same place during imaging. First, the use of the lock-and-key mechanism and the titanium plate, which attaches to the microscope stage, means that the mouse will be located at the same location for each imaging session if returned to the same stage coordinates each time. It also keeps the size of the window to a minimum and maintains accurate positioning over repeated imaging sessions^{263,264}. Together, with the use of fixed reference points from which co-ordinates in the x and y plane are applied, this will ensure return to the same position over repeated imaging sessions. These reference points can include sutures²⁶⁵, or small dots on the window. We also created 'vasculature maps' that helped to confirm the correct area. Another recent paper demonstrated the use of a tile-based approach with a grid printed on the coverslip that aided in returning to the

same position during imaging. Moreover, it facilitated tissue-wide visualization of the calvaria whilst maintaining the single cell resolution²⁶⁴.

The final challenge of intravital imaging windows is the biocompatibility of the window with the mice. This includes the design of the window, which should have limited hindrance to the mouse, including the movements. The calvaria has very little effect on the movement of the mice, and whilst the mice experience some hindrance to movement after surgical implantation of the femur window, this is attenuated after post-surgical swelling is resolved¹⁷⁵. Anti-inflammatory painkillers are administered to the mice post-surgically to promote healing. The windows must also be light weight, biocompatible, non-reactive without causing inflammation, strong, corrosion resistant, reusable and autoclavable for sterilisation. Titanium alloy meets these requirements and is therefore the most frequently used material with these properties. Notably, immunohistochemistry and white blood counts analysis for inflammatory cells did not previously show any signs of an inflammatory response locally or systemically²⁶⁶. For these reasons we produced both the femur window and calvaria window in titanium using high precision printing so that the surface is smooth and prevents any abrasion to the skin. They must also be designed in a way that the mice cannot bite or pull on the sutures as this would threaten the security of the window and risk infection and subsequent adverse effects. We therefore secured both the femur and calvaria window using glue and dental cement with the option of sutures to reinforce the attachment of the window.

Together, these design aspects and properties of both the femur and calvaria window have generated a novel intravital imaging technique that will enable live microscopic imaging of the bone metastasis in real time. Previous studies have used similar cranial windows to image interactions with the vasculature during extravasation in brain metastasis²⁶¹, as well as windows to image the interactions of myeloid cells in the vasculature during the seeding process of lung metastasis²¹². To our knowledge, this is the first femur window developed to image tumour-stromal interactions in the long bone. This will not only permit imaging of the dynamic tumour-stromal interactions during tumour cell seeding, but also reveal many other processes involved in tumour development, including recruitment of the cells within the tumour microenvironment cells as well as invasion and metastasis of the tumour cells.

3.3.2.2 Tissue clearance

We have also developed a tissue clearance technique to image the bone in three dimensions and involved optimisation of previous techniques to generate a protocol suitable to our imaging set up. The overall purpose of tissue clearance is to make the sample as transparent as possible to match the refractive index of the imaging mounting media. The equilibration of the refractive index throughout the tissue, prevents, or at least reduces the scattering of light and renders the sample effectively transparent for direct imaging. There are several key steps to achieve this. The sample must first be dehydrated and delipidated to remove water and lipids, respectively. Water has a low refractive index compared to cellular structure and is the main source of scattering in tissues. Dehydration is most commonly achieved using alcohols, mainly methanol supplemented with or without hexane or tetrahydrofurane, which both remove water as well as some of the lipids. Proteins and lipids also have a high refractive index (1.33 and 1.45, respectively), which results in light scattering. Other components of tissues, such as heme, lipofuscin and melanin pigments, block transmission of light. This is particularly severe in bone and dental tissues due to calcified mineral and collagen, which also block the light. Bone samples must therefore be decolourised to remove the pigments and decalcified to remove the calcium phosphate. The tissue is then immersed in optical clearing agents to finally eliminate the mismatch of refractive index within the tissue.

Several tissue clearance protocols have been developed using a variety of clearing reagents and the efficiency of the reagent is dependent on the tissue type. The basis of the tissue clearance include: hydrophobic methods, which use solvent-based chemicals with high refractive index ($RI < 1.50$), such as BA:BB; hydrophilic methods using aqueous reagents with a lower refractive index ($RI < 1.49$) such as sucrose; and hydrogel-based methods, although the latter was not considered in our project due to the specialist equipment. The advantages and disadvantages of each protocol were compared to identify the one suitable to use for bone metastasis.

Hydrophobic-based clearing approaches require dehydration prior to clearing, therefore as water molecules are required to maintain the emission of fluorescent proteins, their removal from the sample results in poorer signal. This quenching of the fluorescent protein limits their utility. In contrast, some hydrophilic techniques using sugar or glycerol-based reagents developed to overcome the quenching issues of the hydrophobic techniques, do not require delipidation or dehydration and are therefore

less damaging to fluorescent proteins. Hydrophobic-based methods also use toxic chemicals that are not compatible with standard objective lenses as they dissolve glue making imaging more complicated. However, hydrophobic techniques achieve better transparency and hydrophilic clearance is optimal for tissues with little ECM as this does not clear well. Hydrophobic clearing agents preserve the specimen over time as the cleared tissues are hardened. This is advantageous as it allows repeated imaging sessions on a long-term basis, but tissues can also become brittle. Another issue with tissue clearance is that it can either expand or shrink the tissue in hydrophilic and hydrophobic, respectively, and must be considered during analysis and quantification as it can distort distances^{235,267}.

The bone is particularly challenging to clear due to the high density ECM and collagen deposition²⁶⁷. We therefore initially chose to use a hydrophobic-based method to achieve clearance. BA:BB is the most commonly used hydrophobic clearing reagent and is one of the most successful methods used to clear ECM-rich tissues and is particularly favourable for connective tissue²⁶⁷. BA:BB, consisting of 2 parts benzyl benzoate to 1 part benzyl alcohol, was first reported in 2007 as a solvent-based clearance reagent to clear mouse brains and embryos, and fruit flies. It has since been demonstrated to efficiently clear most tissues, including bones. Whilst it is compatible for most immunofluorescent staining, it also quenches fluorescent proteins therefore additional labelling must be used for tissue from reporter mice. Labelling artefacts have also been reported²⁶⁸. BA:BB-based methods have been further adapted to improve the clearance efficiency and preserve fluorescent expression by the addition of further chemicals. Erturk *et al.*²⁶⁹ developed the BA:BB-based protocol, known as three-dimensional imaging of solvent-cleared organs (3DISCO), to overcome the issues with fluorescence loss. The use of tetrahydrofuran in this protocol preserves fluorescent proteins, which overcomes decreased fluorescence emission after dehydration seen in previous BA:BB based methods. However, 3DISCO eventually quenches endogenously expressed fluorescence proteins with a half-life of only a few days leaving limited time for image acquisition^{269,270}. Further changes including the addition of trimethylamine to increase the pH above 9.5 and tert-butanol to dehydrate has overcome the loss of fluorescent proteins, creating a technique known as uDISCO, which has been used utilised in the studies of many diseases including cancer in *in vitro* organoids as well as murine and human samples^{269,271}. This technique has been successfully used for whole body clearing of GFP-labelled mice and permits long term maintenance of fluorescence proteins²⁷². Furthermore, whilst BA:BB clearance results

in tissue shrinkage^{273,274}, this property can be utilised in uDISCO. The samples in uDISCO are reduced in size by up to 65% whilst the anatomical and cellular structures are preserved, which enables subcellular imaging of larger samples and volumes in a single scan therefore reducing artefacts of mosaic imaging¹⁷². This was particularly helpful in identifying and quantifying metastasis as it permits the observing the entire lung and thus the all of the metastasis²⁷⁵. Furthermore, the tumour microenvironment is a heterogeneous with intratumoral variability therefore the tissue clearance of the entire organ permits observation of the differential areas of the tumour^{276,277}.

Together, these adaptations generated a protocol suitable for image the whole bones and thus was initially used. Bones were collected from perfused-fixed mice, and immunofluorescently stained as in the whole mount staining used previously in the lab. The bones were decalcified in EDTA and dehydrated in increasing tert-butanol concentrations before being transferred to BA:BB. Despite the perfusion-fixation, the bones retained a brown colourisation and imaging of the bone marrow could not be achieved with a limited imaging depth of <50µm. This partial success on clearing hard tissue has been reported by others¹⁷². Jing *et al.*¹⁷³ recently improved this technique by replacing BA with PEG, which rendered the majority of tissue types, including the bone, transparent whilst retaining endogenous fluorescence¹⁷³. However, this protocol achieved limited success with the bones in our project.

A hydrophilic-based clearing technique, known as BoneCLARITY, was recently used to successfully clear the bone and detect, image and quantify osteoprogenitor cells²⁷⁸. However, CLARITY is a long process (approximately 1 month), technically challenging and has limited access due to the requirement of specific equipment²⁷⁹. Nevertheless, due to the success of others using hydrophilic-based hydrogels, we decided to use a novel commercially available hydrophilic clearance reagent, known as RapiClear®.

Rapiclear® is a commercial hydrophilic-based tissue clearance reagent, which permits tissue clearance in one step by directly transferring the samples to the clearing reagent immediately after immunofluorescent staining. It is compatible with the majority of endogenous fluorescent proteins, lipophilic tracers and Alexa Fluor dyes, and has been demonstrated in many tissue types, including the bone. Furthermore, the clearance effect is reversible by removal from the reagent if required¹⁷⁴. This is a much less complicated and shorter protocol than those used previously, and due to the commercial manufacture of the product, there is limited variation between batches. In

contrast, previous tissue clearance reagents including BA:BB and BB:PEG involve long, complex protocols sensitive to variables including pH and temperature that can affect the success of the clearance efficiency. Differences between batches may therefore cause variation in fluorescence intensities, for example.

Bones were collected from perfused-fixed mice as before, but were additionally shaved using a cryostat to reduce the depth of the cortical bone, which reduced the distance to the bone marrow for imaging^{280,281}. Immunofluorescently stained bones were immersed in RapiClear1.52® overnight before imaging in the imaging chamber. Another challenge of imaging cleared tissues is that the mounting medium must match the refractive index of the tissue and therefore must be mounted in the clearance medium. These are often damaging to the microscope lenses, and thus must be mounted in special methods. The use of the imaging chamber composed of iSpacers® and coverslips permitted imaging of the bone with little loss of working distance to the chamber.

Multiphoton microscopy is the preferred method for imaging cleared tissues. This is due to the requirement of a high working distance for imaging the cleared tissues and is often a limiting factor preventing deep imaging. Most high numerical aperture objectives, which determine a high resolution, have a relatively short working distance of less than 200µm. This inversely proportional balance between high resolution and working distance creates a challenge in imaging deep tissues at a high resolution. Multiphoton microscopy partly overcomes this issue allowing imaging of almost 1mm deep tissue. The use of second generation harmonics also adds the imaging of collagen without additional staining²³⁵. Specific objectives have also now been manufactured specifically for tissue clearance imaging. These have long working distances over 5mm and a high numerical aperture meaning high resolution images can be acquired of deep issues. However, not only are these objectives expensive and thus unavailable to many labs, images acquired for deep regions still have reduced quality due to excitation of fluorophores outside the focal point. This would be overcome by the pinhole in confocal microscopy, which would also improve signal-to-noise and have faster acquisition times. However, low working distance and limited penetration restrict the use of confocal microscopy for tissue clearance²³⁵. Due to technical issues, we were unable to image the bones using multiphoton microscopy and instead used the Andor Dragonfly Confocal microscope. Imaging depths of

>200µm were achieved with further expected using multiphoton microscopy in the future.

To conclude, the use of the RapiClear1.52® to clear immunofluorescently stained bones permitted successful imaging of the entire bone marrow at a cellular resolution (figure 3.2.19). We have successfully developed and optimised a tissue clearance protocol deep imaging of bone metastasis in the marrow region and enables visualisation of cellular structures and interactions in three-dimensions. This permits measurements and quantification with increased accuracy and precision compared with other imaging techniques²⁷⁸. This technique is now ready to be routinely utilised to image processes occurring in bone metastasis, including accurate measurements of the distance between tumour cells and the niche cells during the seeding period.

3.4 Conclusion

To conclude, we have demonstrated that macrophages play a key role in the seeding and colonisation of breast cancer cells in the bone during metastasis. Macrophage depletion during the seeding period results in reduced surviving tumour cells at the point of seeding, delayed bone metastatic growth and prolonged survival compared to non-depleted controls. The molecular mechanism involved also requires further investigation, whilst preliminary data demonstrates that macrophage-derived VEGFA does not contribute. This is suggestive of a bone-specific mechanism compared to seeding during lung metastasis where macrophage-derived VEGFA promotes seeding. It also remains to be elucidated whether the tumour cells directly seed to a niche derived from macrophages, or whether the macrophages promote seeding to the osteoblasts or MSCs in the endosteal or perivascular HSC niches, previously identified as an important niche for tumour cell seeding in the bone. We have used whole mount staining to provide preliminary data that the bone metastatic breast cancer cells seed to the osteoblast-rich region, but requires confirmation in SP7-GFP mice. It also validates that we can visualise the single cells seeding within the bone. We will combine this with the two novel imaging platforms, intravital imaging and tissue clearance, which will enable real-time and three-dimensional visualisation of the tumour cell seeding to the stromal cells at the kinetic level. This will allow monitoring of the extravasation process and quantification of tumour-stromal interactions in both control and macrophage depleted mice offering further insight into the role of macrophages in tumour cell seeding to the bone.

Chapter 4 Mesenchymal stromal cells in the growth of breast cancer bone metastasis

4.1 Introduction

One of the most abundant components of the TME are mesenchymal stromal cells, consisting of MSCs, pericytes CAFs as well as osteoblasts in the bone. There is now increasing evidence that these cells promote tumour growth via numerous potential mechanisms. This includes the secretion of growth factors that act directly on the tumour cells to promote tumour growth, and the production of pro-angiogenic factors that stimulate angiogenesis within the tumour, which facilitates delivery of nutrients and oxygen, particularly as it increases in size. MSCs also interact with the immune compartment of the TME to create an immunosuppressive environment that permits tumour growth. However, many of these studies were previously performed in models of primary cancer, with limited research into the role of the mesenchymal lineage within bone metastasis.

We therefore aim to investigate the role of the mesenchymal compartment in breast cancer bone metastatic growth (figure 4.1.1). We first aim to characterise the mesenchymal lineage in breast cancer bone metastasis using immunostaining and will subsequently utilise transgenic *in vivo* models to study the effect of stromal cell depletion on bone metastasis growth, including Nestin⁺ MSCs and SP7⁺ osteoblasts. This is complemented with *in vitro* proliferation assays to further confirm the effect of the stromal cells on tumour cell growth. We next begin to identify potential mechanisms downstream of the MSCs to investigate how MSCs regulate tumour growth. We explore several avenues including secretion of growth factors that directly act on the bone metastatic cells to induce growth. We also investigate whether MSCs may undergo differentiation into CAFs within the TME, which in turn promote growth. MSCs may also exert tumour-promoting effects via interactions with TAMs to create an immunosuppressive, pro-tumoral phenotype. Another project has identified a distinct population of macrophages within breast cancer bone metastasis, defined as bone metastasis-associated macrophages (BoMAMs). The depletion of these cells results in

attenuated growth of the bone metastasis. This population also exhibit a specific expression profile, and therefore we will analyse the effects of MSC on the expression of these markers in MSCs, which will determine whether MSCs may promote growth via the induction of these tumour-promoting macrophages.

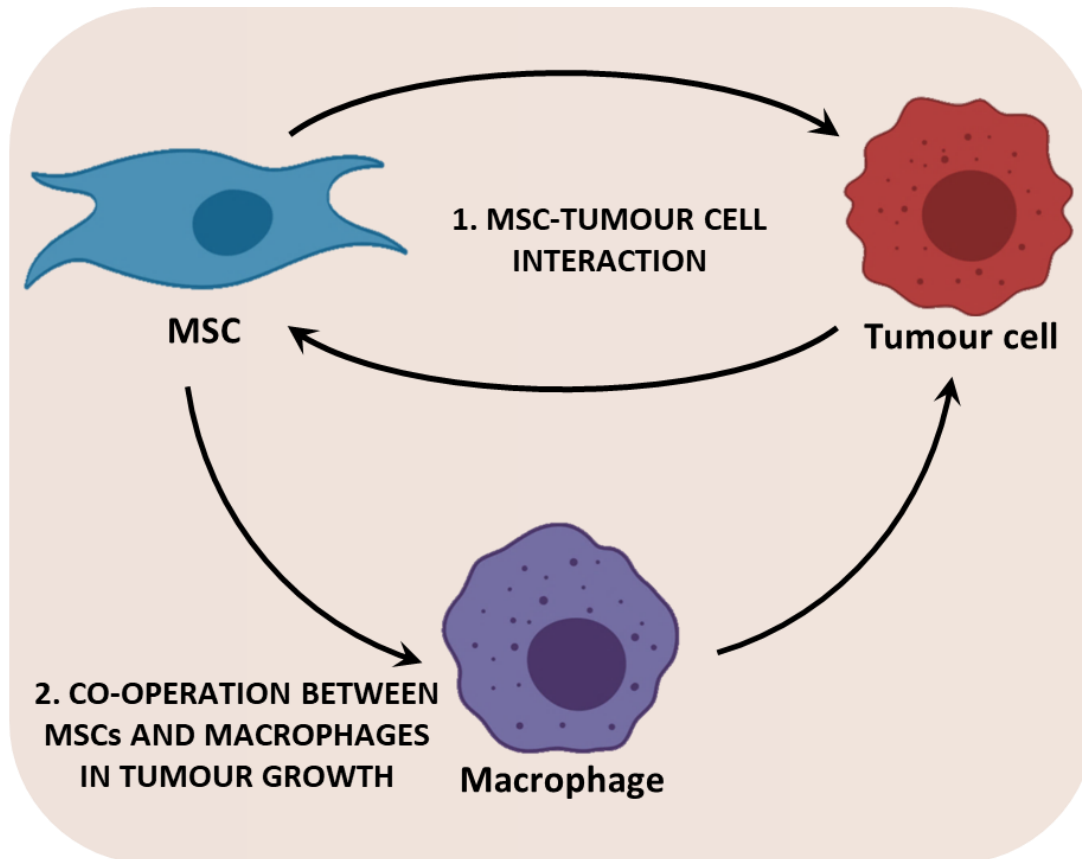


Figure 4.1.1: Potential effects of MSCs on bone metastatic growth. MSCs may mediate tumour growth via direct interactions with the tumour cells, as well as interactions with other components of the TME, including macrophages.

Tumour cells establish paracrine loops with the mesenchymal compartment of the TME to promote these pro-tumorigenic effects. MSCs are recruited to tumour regions via the secretion of chemoattractants from the tumour where they may also proliferate. We therefore aim to determine the effect of bone metastatic tumour cells on the mesenchymal compartment, including proliferation and migration. This will give insight into the tumour-promoting paracrine loops that exist within breast cancer bone metastasis.

4.2 Results

4.2.1 Mesenchymal stromal cells promote bone metastatic growth

To determine the role of MSCs in bone metastatic growth, we generated bone metastasis via intracardiac injection of MetBo2 cells in Nestin-cre^{ERT2}-iDTR-GFP-nude mice. These mice were generated by crossing of C57BL/6-Tg(Nes-EGFP), C57BL/6-Tg(Nes-cre/ERT2)KEisc/, Gt(ROSA)26Sortm1(HBEGF)Awai/J (iDTR), and Foxn1nu/Crl. The C57BL/6-Tg(Nes-EGFP) mice express GFP under the regulatory element of the *Nestin* promoter¹⁷¹ therefore label the MSCs *in vivo*, which allowed us to investigate the infiltration of MSCs within bone metastasis. Whole mount staining was utilised to visualise the nestin⁺ MSC content in control and metastatic bones. Breast cancer bone metastasis bones were infiltrated with an increased Nestin⁺ MSCs, whereas these cells were confined to the blood vessels in control bones (figure 4.2.1a, figure 4.2.1b). This was confirmed using injection of anti-CD31 antibody prior to the sacrifice of the mice to label the blood vessels. Nestin⁺-GFP was located along the major blood vessels in the marrow (figure 4.2.1b).

These mice also facilitate the conditional depletion of nestin⁺ MSCs. The injection of tamoxifen via intraperitoneal injection induces Cre expression in Nestin-expressing cells, which cleaves the loxP sites and removes the STOP codon. This induces expression of diphtheria toxin receptor on the surface of Nestin⁺ cells. Subsequent administration of diphtheria toxin (or mutated Glu⁵² diphtheria toxin in controls) results in conditional ablation of Nestin⁺ MSCs (figure 4.2.2a). To determine the effect of nestin⁺ MSC depletion on bone metastatic growth, we generated bone metastasis via the intrailliac injection of luciferase-labelled MetBo2 cells. Once bone metastases were detected using bioluminescence imaging, we depleted the nestin⁺ MSCs using this protocol (figure 4.2.2b). Non-depleted control mice received Glu⁵²DT. Bioluminescence imaging was used to monitor tumour growth throughout the depletion. Whole mount staining of bones collected on day 7 of depletion confirms that this model results in efficient Nestin⁺ MSC depletion in comparison to non-depleted controls in the tumour region with a significant reduction in nestin-GFP area observed in depleted tumours compared to non-depleted controls (figure 4.2.3). In contrast, normal regions of the bone, including the bone marrow and growth plate/epiphysis, did not exhibit effective depletion of the Nestin⁺ MSCs with similar expression patterns observed in both control and depleted mice and no significant difference in nestin-GFP areas (figure 4.2.3).

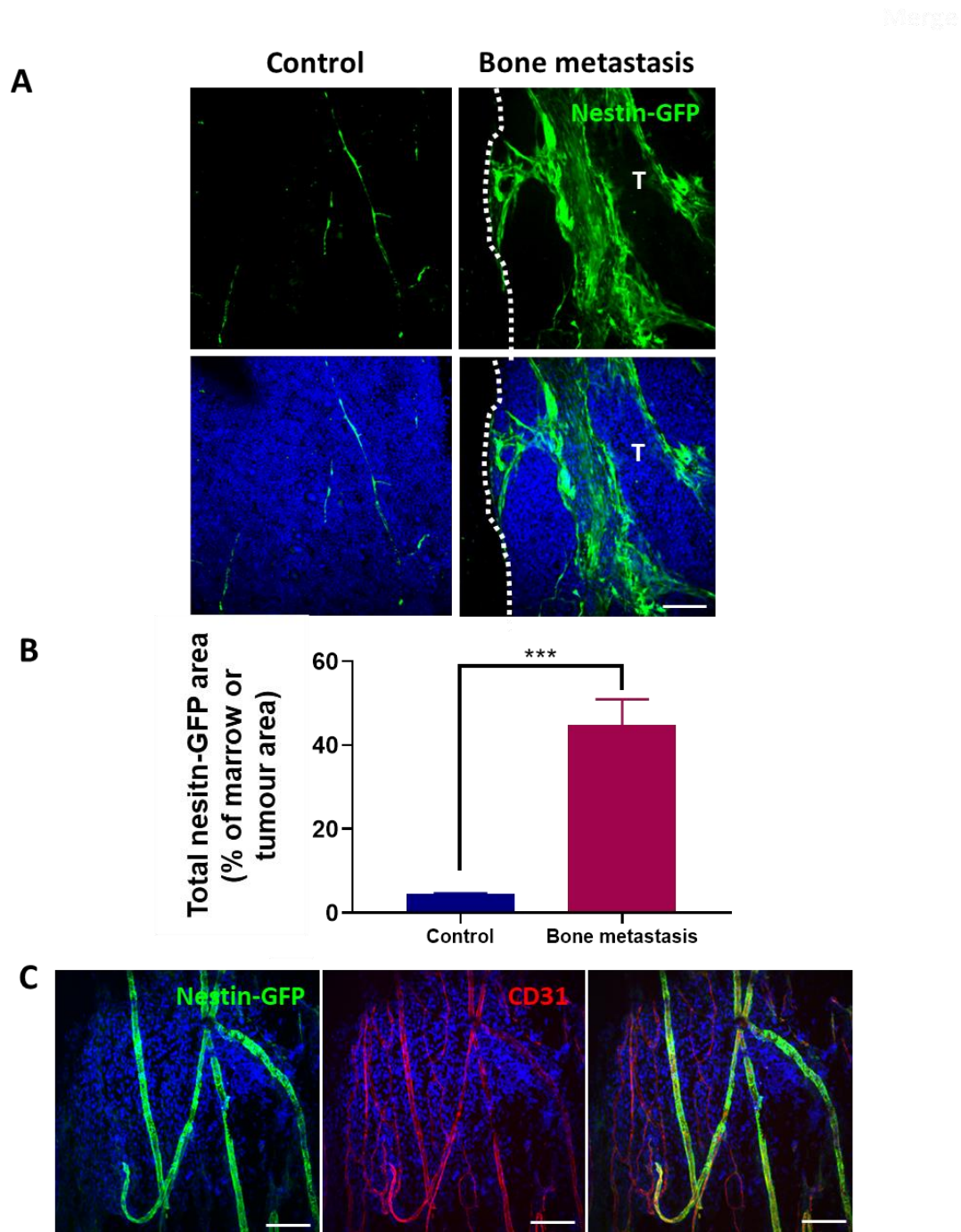


Figure 4.2.1: Abundance of Nestin⁺ MSCs is significantly increased in bone metastasis.
A) Whole mount fluorescent images (maximum projection) of normal bone marrow from non-tumour control mice and MetBo2-derived bone metastasis stained for nestin-GFP (top) and merged with DAPI (bottom). Representative images from 3-4 mice are shown. Scale bar: 100µm. **B)** Quantification of nestin-GFP area in normal bone marrow from non-tumour control mice and bone metastasis from tumour-bearing mice. Data shown are mean±SEM (**p<0.0005, unpaired student's t-test, n=3-4). **C)** Whole mount fluorescent images (maximum projection) of normal bone marrow stained with Nestin-GFP (left), blood vessel marker CD31 (centre) and merged with DAPI (right). Scale bar: 100µm.

The depletion of nestin⁺ MSCs during bone metastatic growth results in a significant reduction in tumour growth compared to non-depleted controls (figure 4.2.4). Tumour size is reduced after 7 days of depletion compared to the start of depletion suggesting nestin⁺ MSC depletion reversed tumour growth and is required for tumour cell survival.

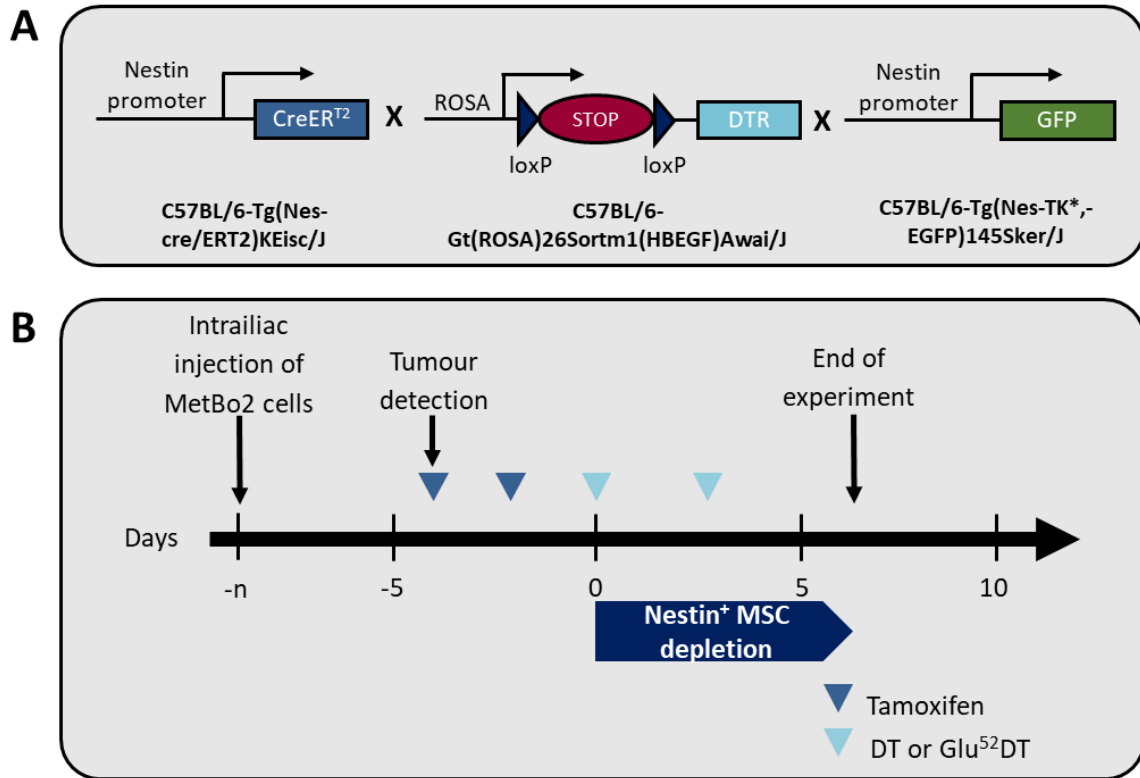


Figure 4.2.2: Effect of Nestin⁺ MSC depletion on bone metastatic growth. **A)** Schematic diagram illustrating the mouse crosses and the genetic details of the strains used to produce the nestin-GFP-Cre-iDTR mouse model. **B)** Experimental design: experimental bone metastasis assay in nestin-GFP-Cre-iDTR mice. Administration of DT induced nestin⁺ MSC depletion, whilst control mice received Glu⁵²DT (DT, diphtheria toxin).

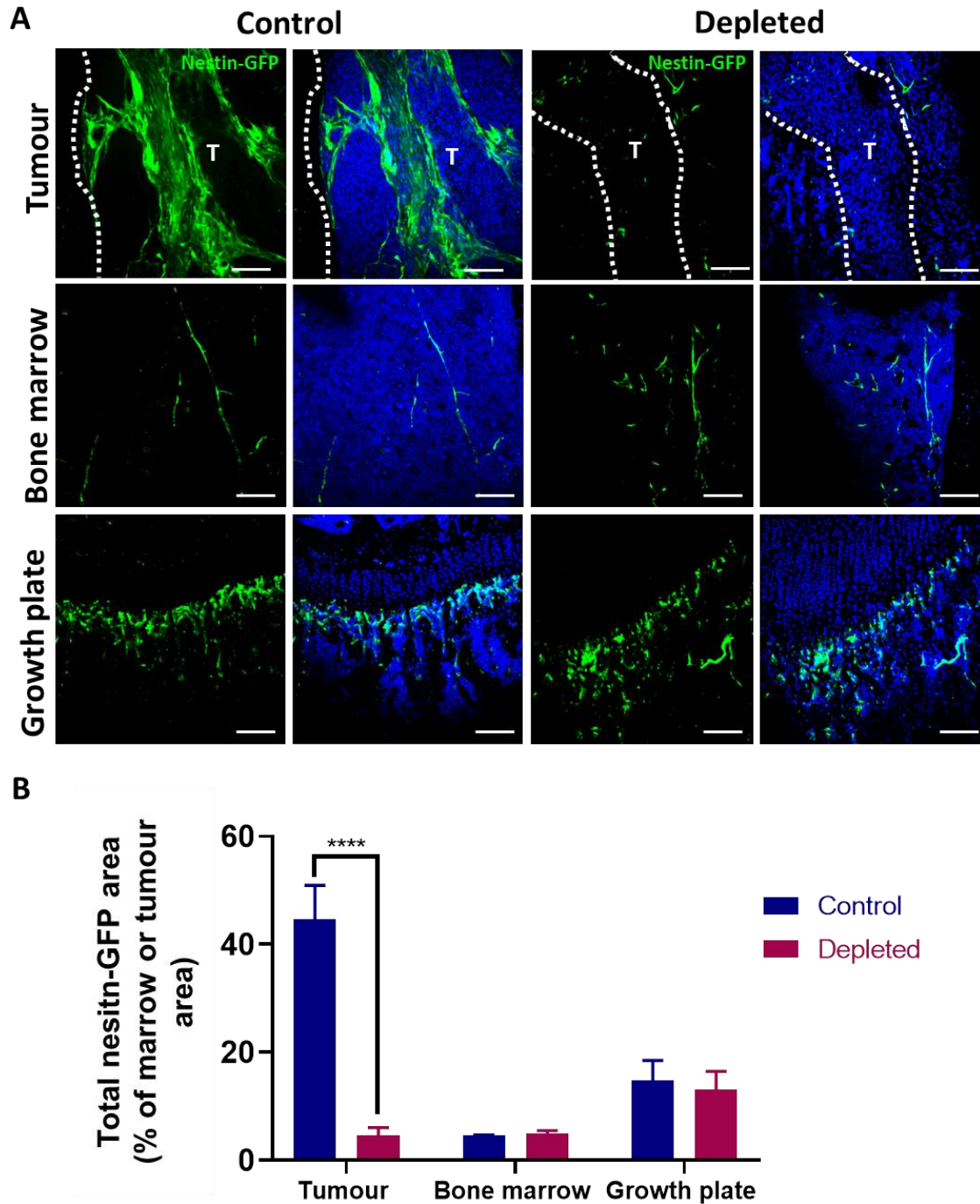


Figure 4.2.3: Efficiency of Nestin⁺ MSC depletion is high in tumour region, but low in normal marrow and growth plate. A) Whole mount fluorescent images (maximum projection) of non-depleted control bones and Nestin⁺ MSC depleted bones stained for nestin-GFP (left) and merged with DAPI (right). Representative images from 3-4 mice are shown. T, tumour area; scale bar: 100µm. **B)** Quantification of nestin-GFP area in normal and tumour-bearing bones collected from control and nestin-depleted mice. Data shown are mean±SEM (****p<0.0001, two-way ANOVA, n=3-4).

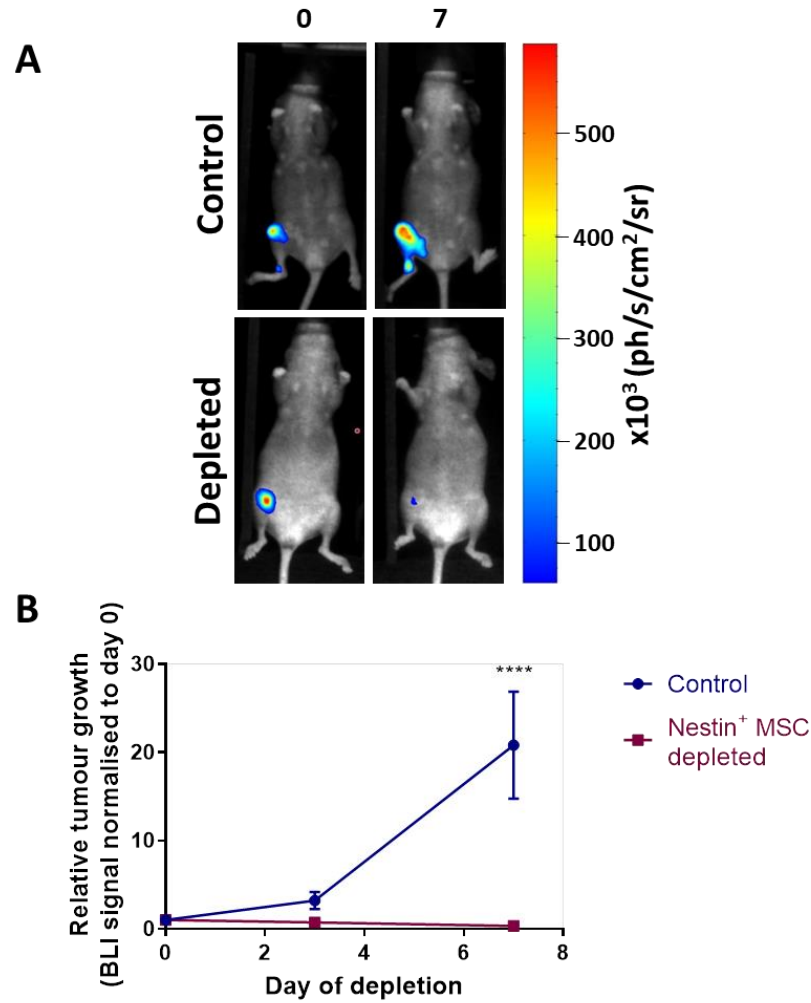


Figure 4.2.4: Nestin⁺ MSC depletion significantly reverses the growth of breast cancer bone metastasis **A)** Representative images of the BLI signal of each chemotherapy group on at start of depletion (day 0) and end point (day 7). **B)** Quantification of bone metastatic growth in control and nestin⁺ MSC depleted mice. Growth was monitored using bioluminescent imaging on day 0, 3 and 7 of depletion. Data shown are mean $\pm$ SEM (****P<0.0001, two-way ANOVA, n=3-4).

We further visualised the mesenchymal compartment in the TME by staining for CD146 in bone metastasis established in FVB/N mice. Whilst CD146⁺ cells were located along the blood vessels in control bones, bone metastasis exhibited an infiltration of CD146⁺ cells through the tumour (figure 4.2.5). CD146 abundance in bone metastasis also positively correlates with proliferation in bone metastasis (figure 4.2.6) supporting that CD146⁺ cells play a role in proliferation and bone metastatic growth. This is not observed in tumours in the mammary fat pad generated from percutaneous injection of MetBo2 cells (figure 4.2.7), indicative of a specific effect of bone metastatic CD146⁺ cells.

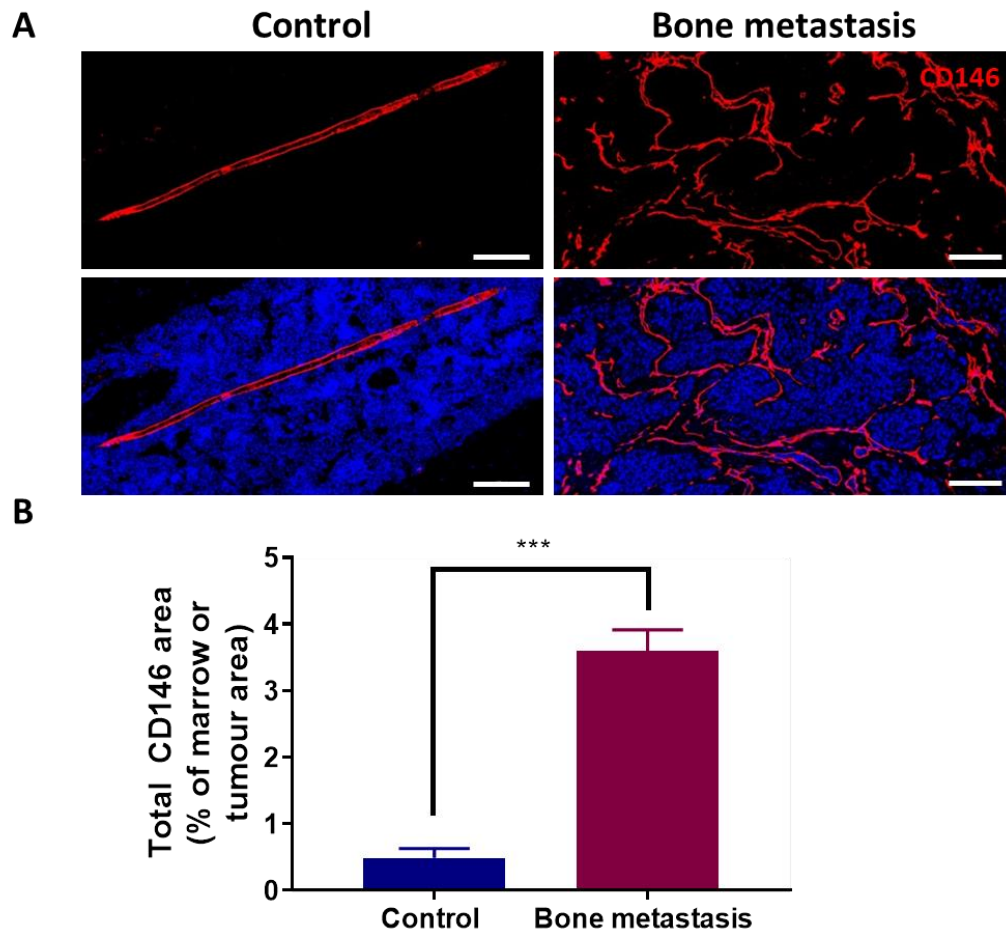


Figure 4.2.5: CD146 abundance is significantly increased in bone metastasis compared to normal control bone. A) Representative immunofluorescence images stained for CD146 (top) and merged with DAPI (bottom) in control and metastatic bones. Scale bar: 100µm. **B)** Quantification of CD146 abundance in normal bone marrow from non-tumour control mice and bone metastasis from tumour-bearing mice. Data shown are mean±SEM (**p<0.0005, unpaired student's t-test, n=3-5).

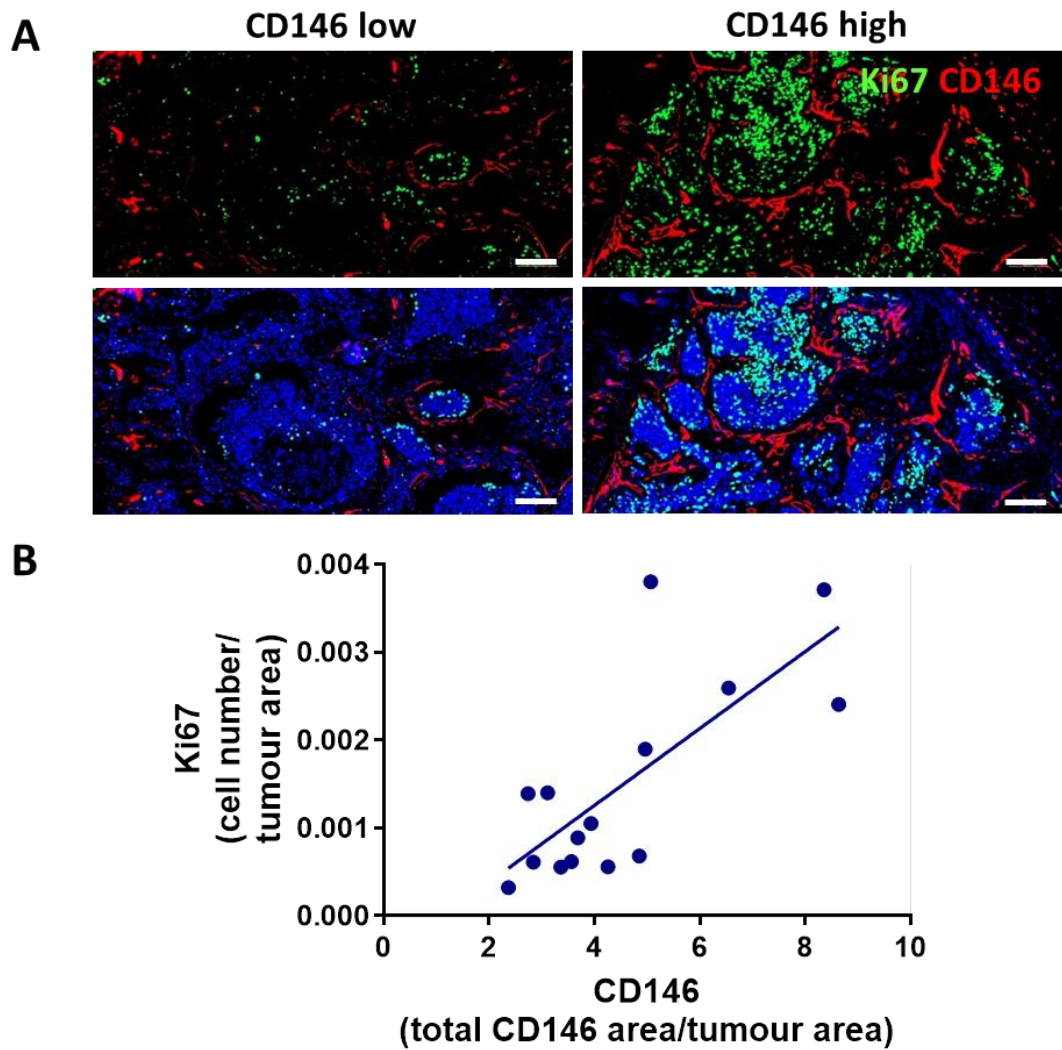


Figure 4.2.6: CD146 abundance positively correlates with proliferation in bone metastasis. **A)** Representative immunofluorescence images stained for CD146 and proliferation marker Ki67 (top) and merged with DAPI (bottom) in CD146-low (left) and CD146-high (right) bone metastasis. Scale bar: 100 μ m. **B)** Correlation between CD146 abundance and number of Ki67⁺ cells in bone metastasis ($r=0.7392$, $r^2=0.5464$, $p=0.0016$, Pearson's correlation, $n=15$).

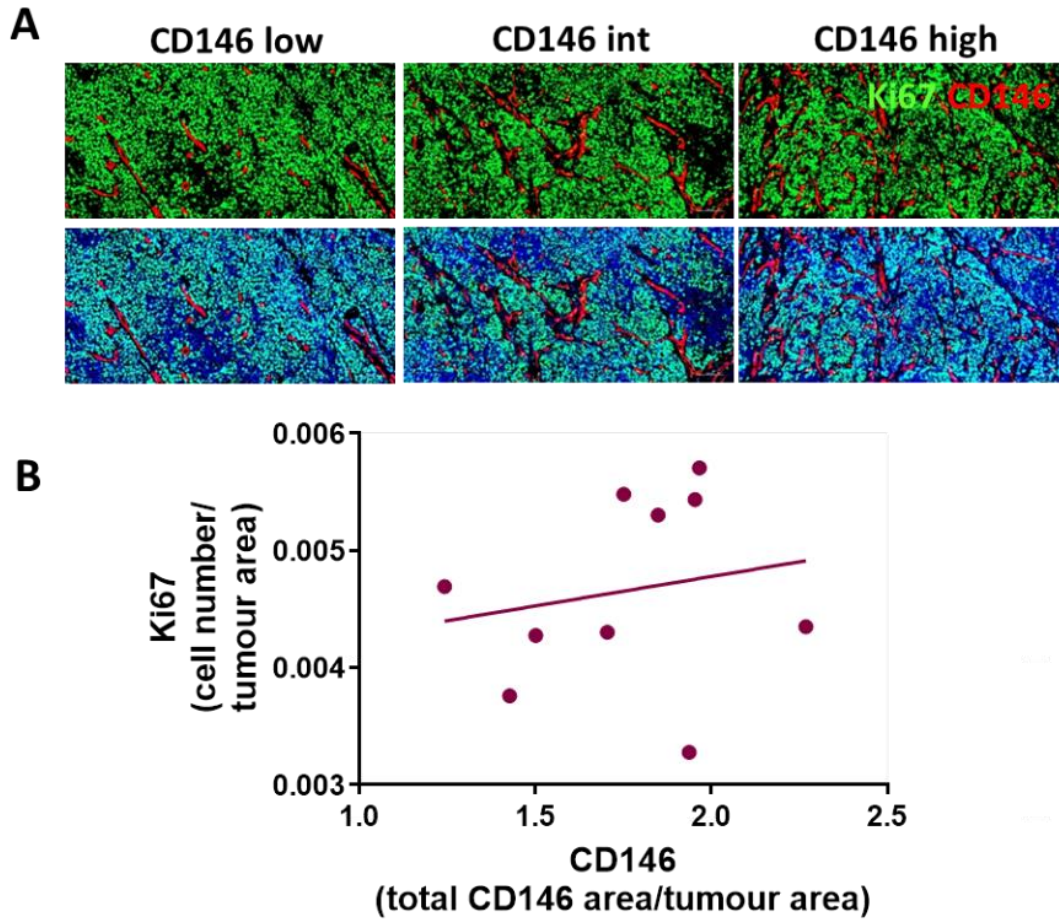


Figure 4.2.7: CD146 abundance does not correlate with proliferation in MFP tumours. A) Representative immunofluorescence images stained for CD146 and proliferation marker Ki67 (top) and merged with DAPI (bottom) in CD146-low (left), CD146-int (centre) and CD146-high (right) MFP tumours. Scale bar: 100µm. **B)** Correlation between CD146 abundance and number of Ki67⁺ cells in MFP tumours ($r=0.1886$, $r^2=0.03556$, $p=0.6019$, Pearson's correlation, $n=10$).

To further support a role of mesenchymal stromal cells in bone metastatic growth, we used *in vitro* proliferation assays. MSCs were isolated from both murine and human bone marrow. These cells were plastic-adherent and exhibited the spindle-like, elongated morphology characteristic of MSCs. Flow cytometry analysis demonstrates that these the cells isolated from murine (figure 4.2.8a) and human bone marrow (figure 4.2.8b) express high levels of MSC markers (Sca1, CD105, CD29 and CD44), whilst expressing low levels of immune cell, endothelial cell and HSC markers (CD45, CD11b, CD14, CD31, CD34). The cells were also able to differentiate into osteoblasts, chondrocytes and adipocytes, as well as fibroblasts defined by alizarin red and alkaline phosphatase (ALP) staining, toluidine blue staining, Oil Red O staining and α SMA

staining. (These differentiation assays were performed by Dr Galadrielle Biver, data not shown). Together, this meets the required criteria confirming that the isolated cells are MSCs³³.

MSCs isolated using these methods were used in *in vitro* proliferation assays. For direct co-culture assays, breast cancer bone metastatic cells were seeded at 500cells/well into 48 well plates either alone or in direct co-culture with MSCs at a MetBo2:MSC ratio of 1:1 to 1:8. Cells were cultured for 3 days after which a luciferase assay was used to measure the number of tumour cells (figure 4.2.9a). The direct co-culture of MetBo2 cells with murine bone-marrow derived MSCs (BM-MSCs) resulted in a MSC-number dependent increase in MetBo2 growth (figure 4.2.9b). The direct co-culture of 1833 cells with human bone marrow derived cells (HBMSCs) also caused an increase in 1833 cell growth, which was significant at a 1:8 ratio (figure 4.2.9c). A similar growth pattern was observed with co-culture of MSC cell line, C3H10T12, albeit non-significant (figure 4.2.9d).

A transwell assay was used to determine the role of MSC-derived soluble factors on bone metastatic growth (figure 4.2.10a). Tumour cells were seeded in 24 well plates at a concentration of 1000cells/well. MSCs were seeded into 0.4µm-pore transwell inserts in a separate plate at a MetBo2:MSC ratio of 1:1-1:8. After 24 hours, transwells were transferred to the wells containing the tumour cells. MSC-derived conditioned media was also used as a variable to allow the effects of soluble components that cannot migrate through the pores to be analysed. The cells were cultured for 3 days after which an MTT assay was used to measure the cell viability of tumour cells. The indirect co-culture of MetBo2 and 1833 cells with murine BM-MSCs and HBMSCs, respectively, had no effect on tumour cell growth (figure 4.2.10b, figure 4.2.10c).

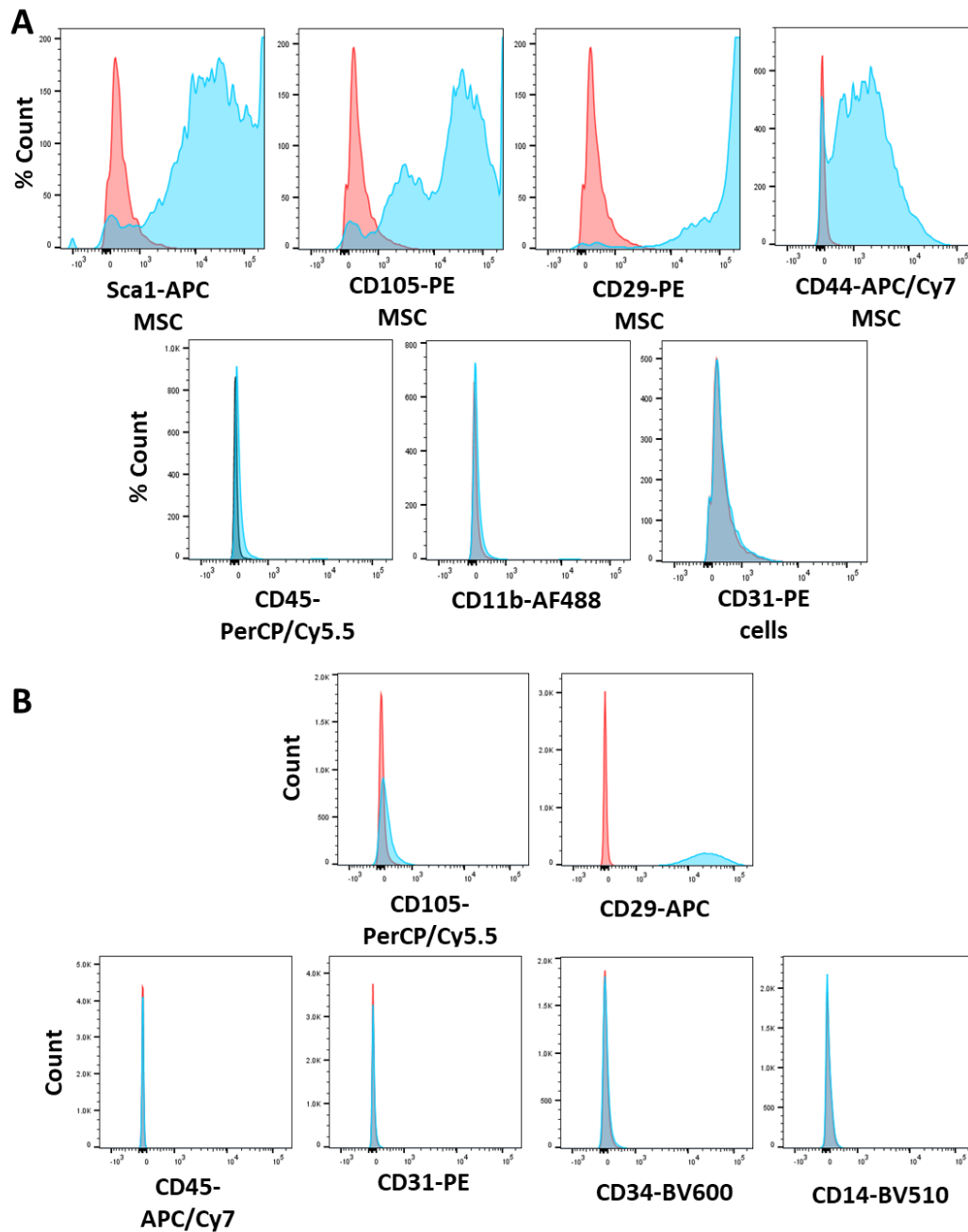


Figure 4.2.8: Characterisation of bone marrow-derived MSCs. Flow cytometry analysis of **A)** murine and **B)** human bone marrow-derived MSCs at passage 3 in *in vitro* culture. Flow cytometry experiment was performed by Dr Galadrielle Biver.

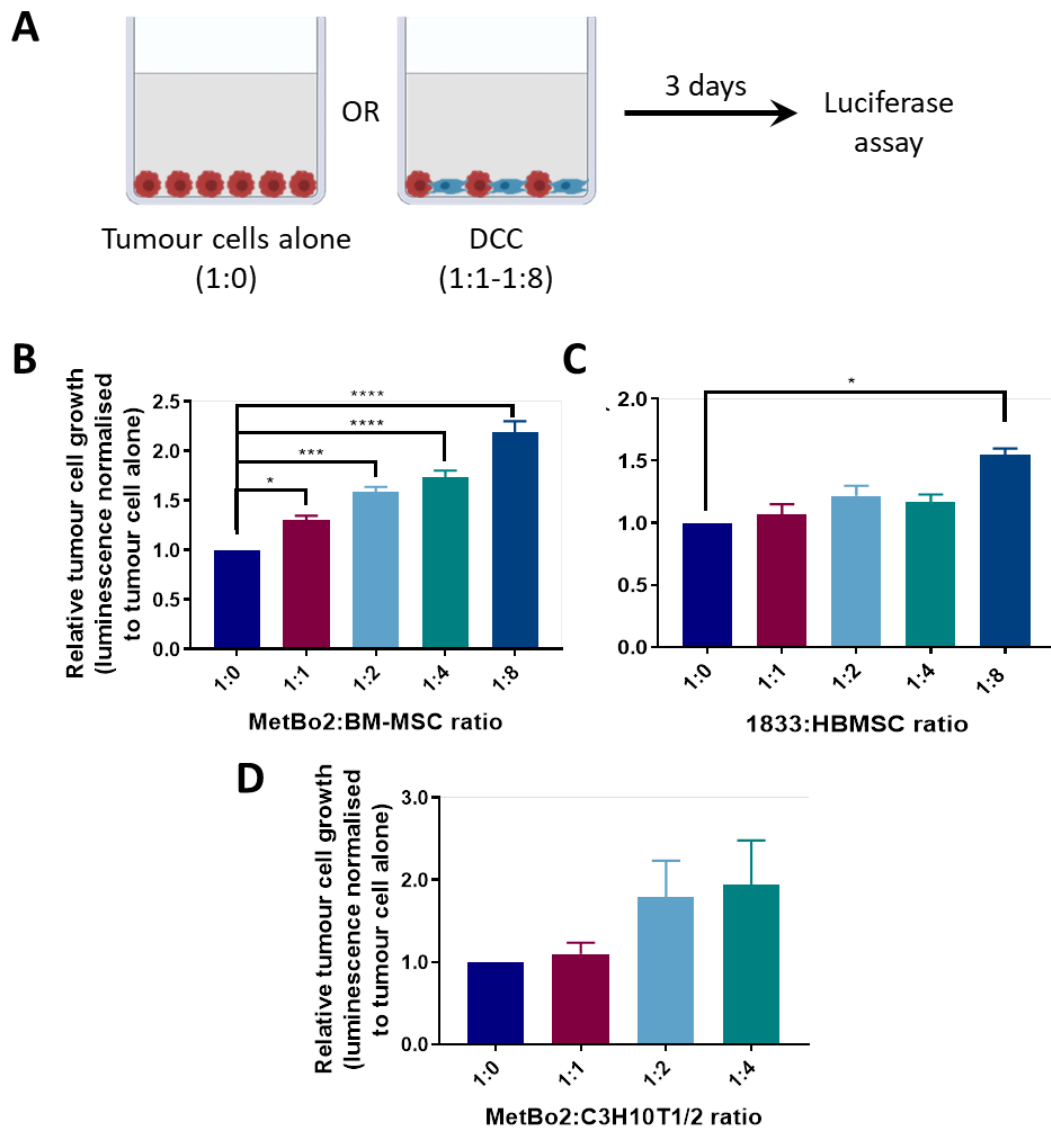


Figure 4.2.9: Direct contact between mesenchymal stromal cells and tumour cells promote tumour cell growth *in vitro*. **A)** Experimental design: proliferation assay of tumour cells alone, or in direct-co-culture with MSCs (Red, tumour cells; blue, MSC; DCC, direct co-culture). **B-D)** Tumour cell growth in culture of **B)** MetBo2 with BM-MSCs, **C)** 1833 with HB-MSCs and **D)** MetBo2 with C3H10T12. Cell growth was quantified by luciferase assay on day 3 of culture. Data shown are mean $\pm$ SEM (* p <0.05 *** p <0.001 **** p <0.0001, one-way ANOVA, n =3).

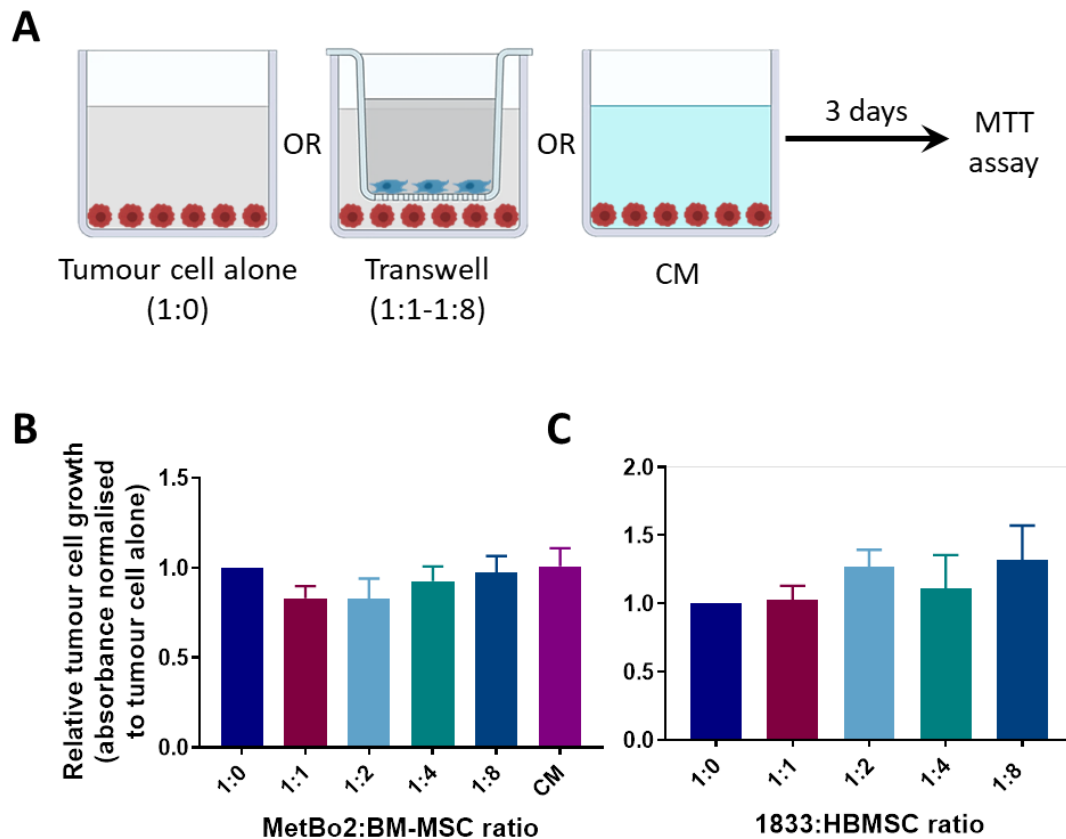


Figure 4.2.10: Secreted factors from mesenchymal stromal cells have no effect on tumour cell growth *in vitro*. **A)** Experimental design: proliferation assay of tumour cells alone or with MSCs in 0.4µm transwells or in MSC-derived conditioned media (red, 1833 cells; blue, MSC). **B-C)** Tumour cell growth in culture of **B)** MetBo2 with BM-MSCs and **C)** 1833 with HB-MSCs. Cell growth was quantified by MTT assay on day 3 of culture. Data shown are mean±SEM (n=3).

These results demonstrate that bone metastases are infiltrated with populations of mesenchymal stromal cells, which promote metastatic growth. We therefore sought to identify the origins of these cells in bone metastasis with a particular focus on proliferation and recruitment. Immunostaining of CD146 and Ki67 revealed that CD146⁺ cells exhibit increased proliferation in bone metastasis compared to normal bone (figure 4.2.11). In bone metastasis 10-15% of total CD146⁺ cells also express Ki67 whilst only ~5% express Ki67 in normal bone. We further investigated proliferation as a potential source of CD146⁺ cells using an *in vitro* assay. Murine bone marrow MSCs were cultured in control media or conditioned media derived from MetBo2 cells. After 3 days, an MTT assay was used to measure the MSC viability (figure 4.2.12a). Increased growth was observed in MSCs cultured in MetBo2-derived conditioned media compared to those cultured in control media (figure 4.2.12b). This suggests that tumour

cell-derived factors may promote MSC proliferation observed in bone metastasis *in vivo*.

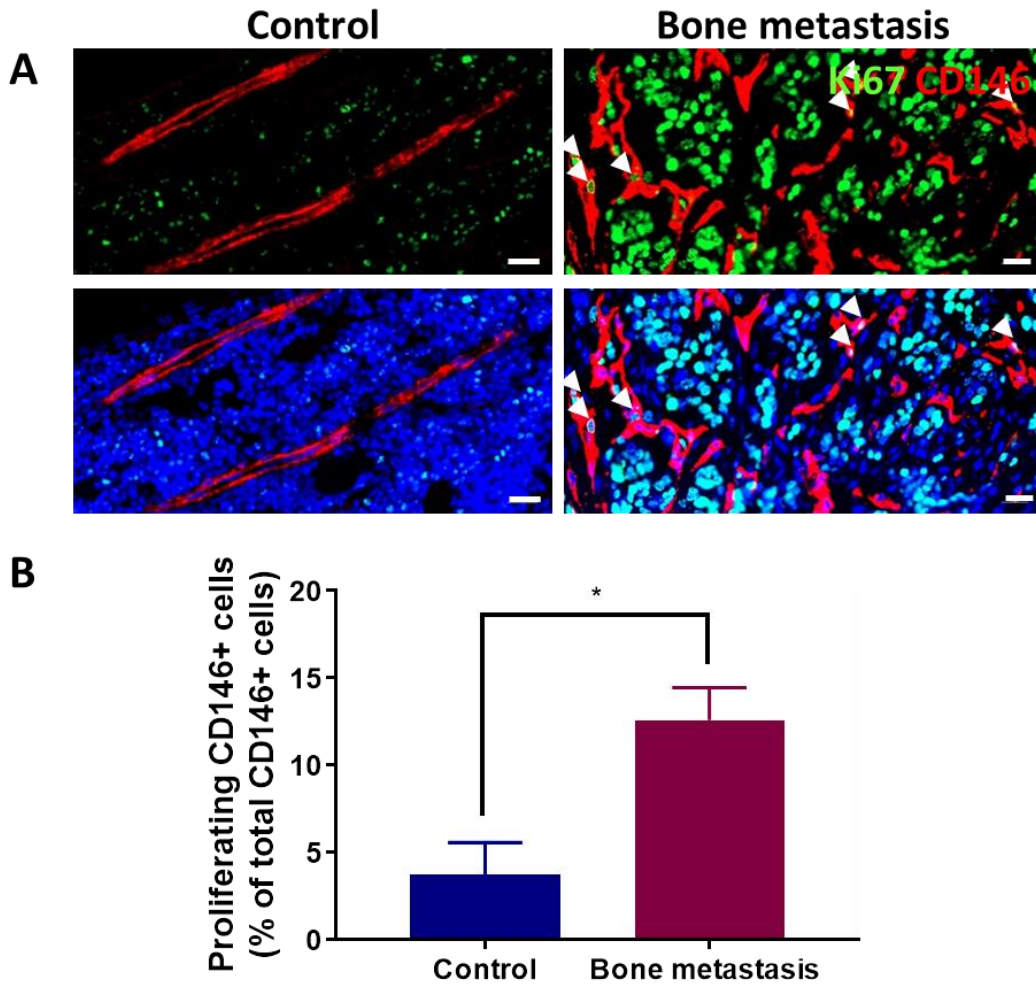


Figure 4.2.11: Cancer-associated MSCs exhibit significantly increased proliferation compared to normal, control MSCs. A) Representative immunofluorescence images stained for CD146 and Ki67 (top) and merged with DAPI (bottom) in control bone (left) and bone metastasis (right). White arrow: double positive cells; scale bar: 20µm. **B)** Quantification of proliferating CD146+ cells in control bone and bone metastasis. The number of proliferating CD146+ cells was calculated by the percentage of CD146+ cells expressing Ki67. Data shown are mean±SEM (*p<0.05, unpaired student's t-test, n=3-7).

Another source of tumour-associated stromal cells is recruitment from other tissues. We thus investigated whether bone metastatic cells can induce the migration of MSCs using *in vitro* migration assays. Bone marrow-derived MSCs were seeded in 8.0µm-pore transwells, which placed in wells containing control media or conditioned media derived from MetBo2 cells. MSCs were left to migrate for 12 hours and stained with

crystal violet to identify migrated cells (figure 4.2.13a). In comparison to control media, MetBo2-derived conditioned media significantly enhanced MSC migration (figure 4.2.13b). This suggests that bone-metastatic tumour cells secrete soluble factors that promote the recruitment of MSCs to the tumour region, resulting in the increased abundance of MSCs observed *in vivo*.

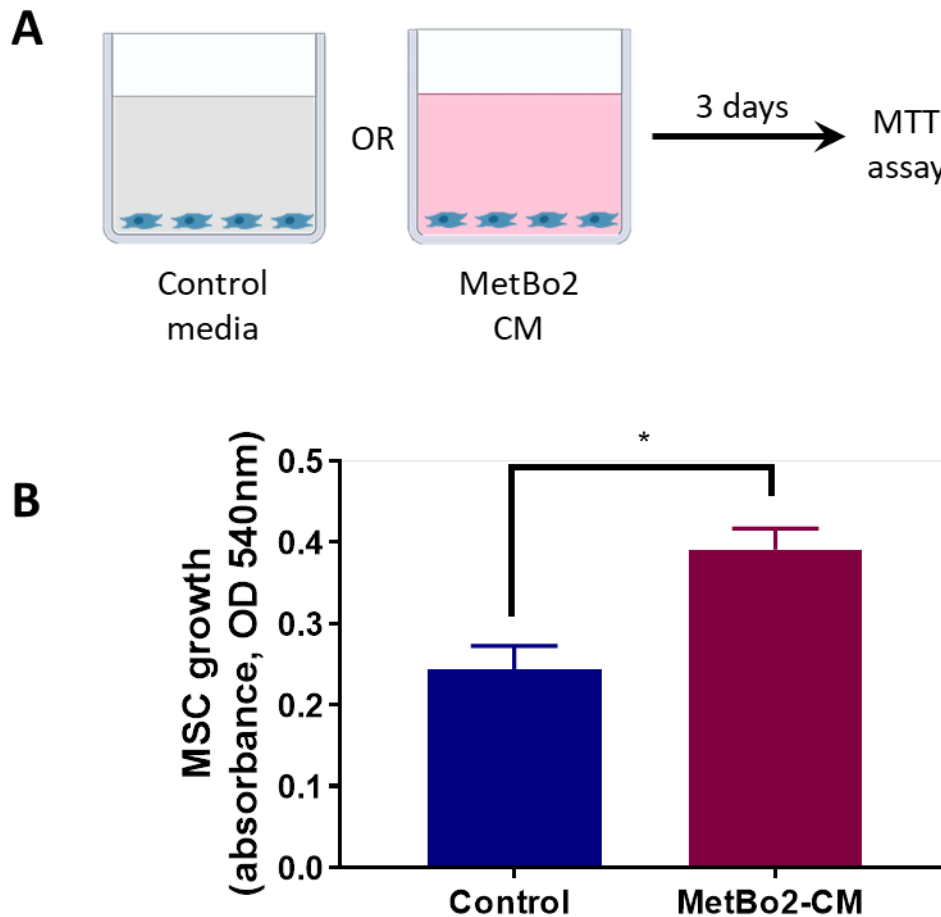


Figure 4.2.12: Tumour cell-derived secreted factors promote MSC proliferation *in vitro*. **A)** Experimental design: proliferation assay of BM-MSCs in control media or conditioned media derived from MetBo2 cells (blue, MSCs; CM, conditioned media). **B)** MSC growth in control and MetBo2-derived conditioned media *in vitro* measured by MTT assay on day 3 of culture. Data shown are mean±SEM (* $p < 0.05$, unpaired student's t-test, $n = 4$).

Together with the effect of MSC depletion, this data suggests that mesenchymal cells, particularly Nestin⁺ MSCs and potentially CD146⁺ MSCs, are important in promoting bone metastatic growth. This effect appears to be dependent on direct contact between the MSCs and tumour cells, whilst MSC-derived soluble factors alone do not have any effect. Moreover, soluble factors secreted from the bone metastatic cells appear to

induce both proliferation and migration of the MSCs establishing a paracrine loop that will contribute to metastatic growth.

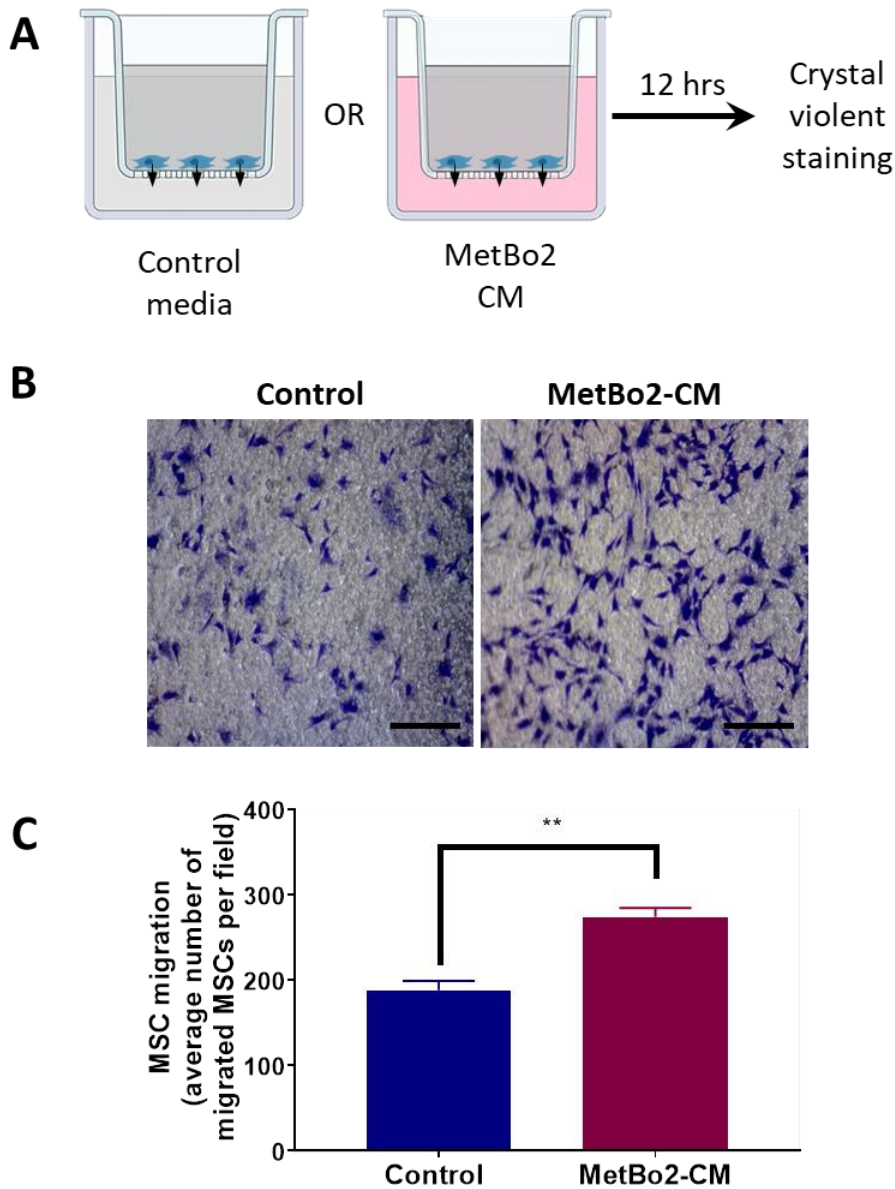


Figure 4.2.13: Tumour-derived secreted factors induces MSC migration *in vitro*. **A)** Experimental design: migration assay of MSCs towards control media or MetBo2-derived conditioned media (blue, MSCs). **B)** Representative images of MSCs in the transwells migrated towards control media (left) or MetBo2-derived conditioned media (right). Scale bar: 100µm. **C)** Quantification of MSC migration across transwells towards control media or CM derived from MetBo2 tumour cells. Data shown are mean±SEM (**p<0.01, unpaired student's t-test, n=4).

4.2.2 MSC-CAF differentiation

Another component of the mesenchymal compartment of the TME is fibroblasts. Previously CAFs have been identified using α SMA²⁸² and therefore was used to stain for similar cells in bone metastasis. Whilst α SMA⁺ cells were confined to the blood vessels in normal bones, the abundance of α SMA⁺ cells were significantly increased in bone metastasis (figure 4.2.14). Using alternative fibroblast markers, we demonstrate that FAP⁺ and FSP⁺ fibroblasts also infiltrate bone metastasis (figure 4.2.15).

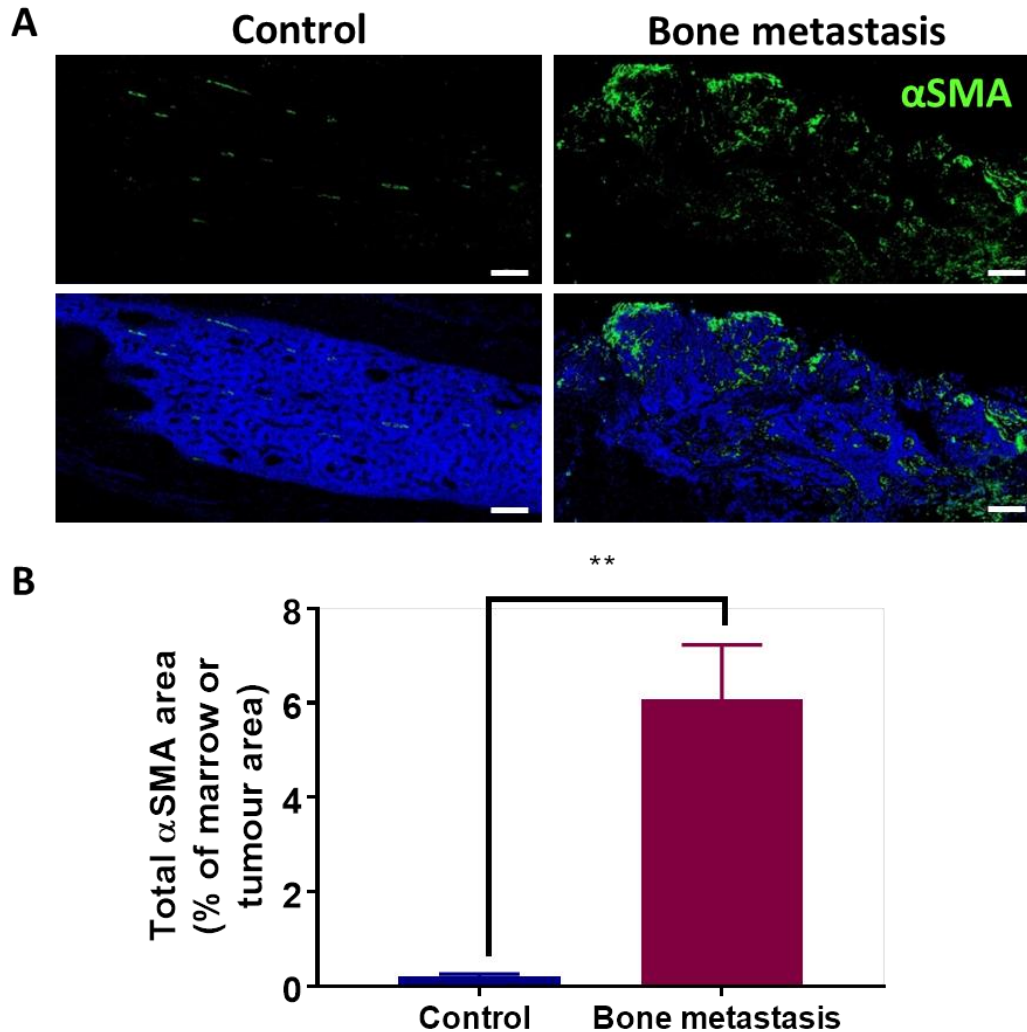


Figure 4.2.14: α SMA abundance is significantly increased in bone metastasis compared to normal control bone. A) Representative immunofluorescence images stained for α SMA (top) and merged with DAPI (bottom) in control and metastatic bones. Scale bar: 200 μ m. **B)** Quantification of α SMA abundance in normal bone marrow from non-tumour control mice and bone metastasis from tumour-bearing mice. Data shown are mean $\pm$ SEM (** p <0.01, unpaired student's t-test, n =3).

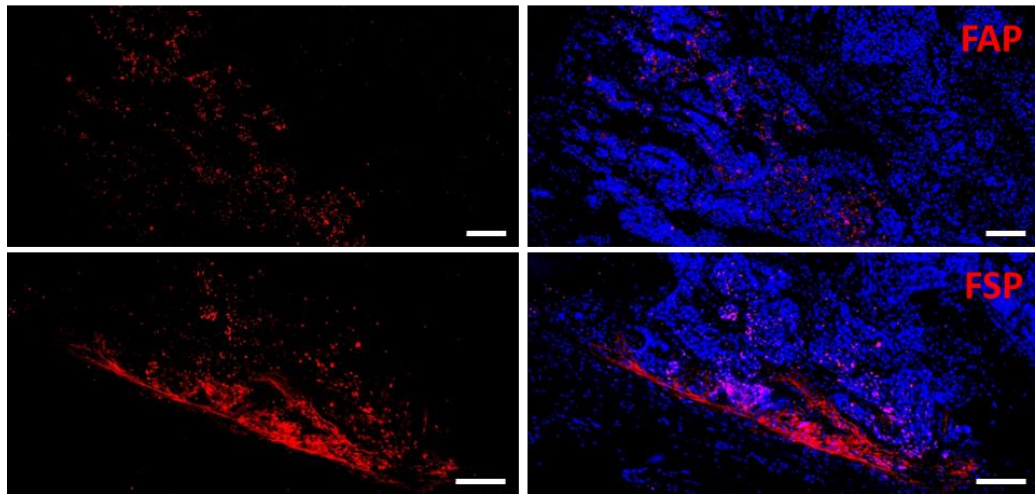


Figure 4.2.15: Fibroblasts infiltrate bone metastasis. Representative immunofluorescence images stained for **A)** FAP and **B)** FSP (left) and merged with DAPI (right) metastatic bones. Scale bar: 100µm. Representative images of 3 and 4 tumours are shown for FAP and FSP, respectively.

We therefore used *in vitro* proliferation assays to determine if α SMA⁺ cells promote bone metastatic growth. Normal fibroblasts (NFs) and cancer-associated fibroblasts (CAFs) were previously isolated from the normal or cancerous breast tissue of patients undergoing surgery. These cells were directly co-cultured with 1833 cells as before (figure 4.2.16a). A CAF-number dependent increase in growth was observed with significance increase at a ratio of 1:4 and 1:8 (figure 4.2.16b). Similarly, direct co-culture of NF with 1833 promoted tumour cell growth, although at limited ratios (figure 4.2.16c, figure 4.2.16d). Comparable results were also observed in indirect co-culture transwells assays of 1833 cells with fibroblasts (figure 4.2.17). Whilst the extent of growth-promoting effects varies between each batch of fibroblasts, this signifies that fibroblasts also promote bone metastatic growth via the secretion of fibroblast-derived soluble factors. Additionally, NFs and CAFs both exhibit growth-promoting effects suggesting the effects are not dependent on a cancer-activated phenotype, although the 1833 cells may activate the NFs in the co-culture.

As fibroblasts were also recruited to bone metastasis, we also investigated whether fibrogenic differentiation of MSCs may serve as a source of these fibroblasts as previously observed¹⁷⁰. We used an *in vitro* differentiation assay to measure the α SMA expression in MSCs cultured for 3 days in control media, conditioned media derived from MetBo2 cells, or with MetBo2 cells (figure 4.2.18a). Staining of MSCs with α SMA reveals that only MSCs in close contact with MetBo2 express α SMA in the direct co-culture, whilst MSCs cultured in MetBo2-derived conditioned media display minimal

expression similar to controls (figure 4.2.18b). This suggests that direct contact between MetBo2 and MSCs may induce differentiation to α SMA⁺ fibroblast-like cells.

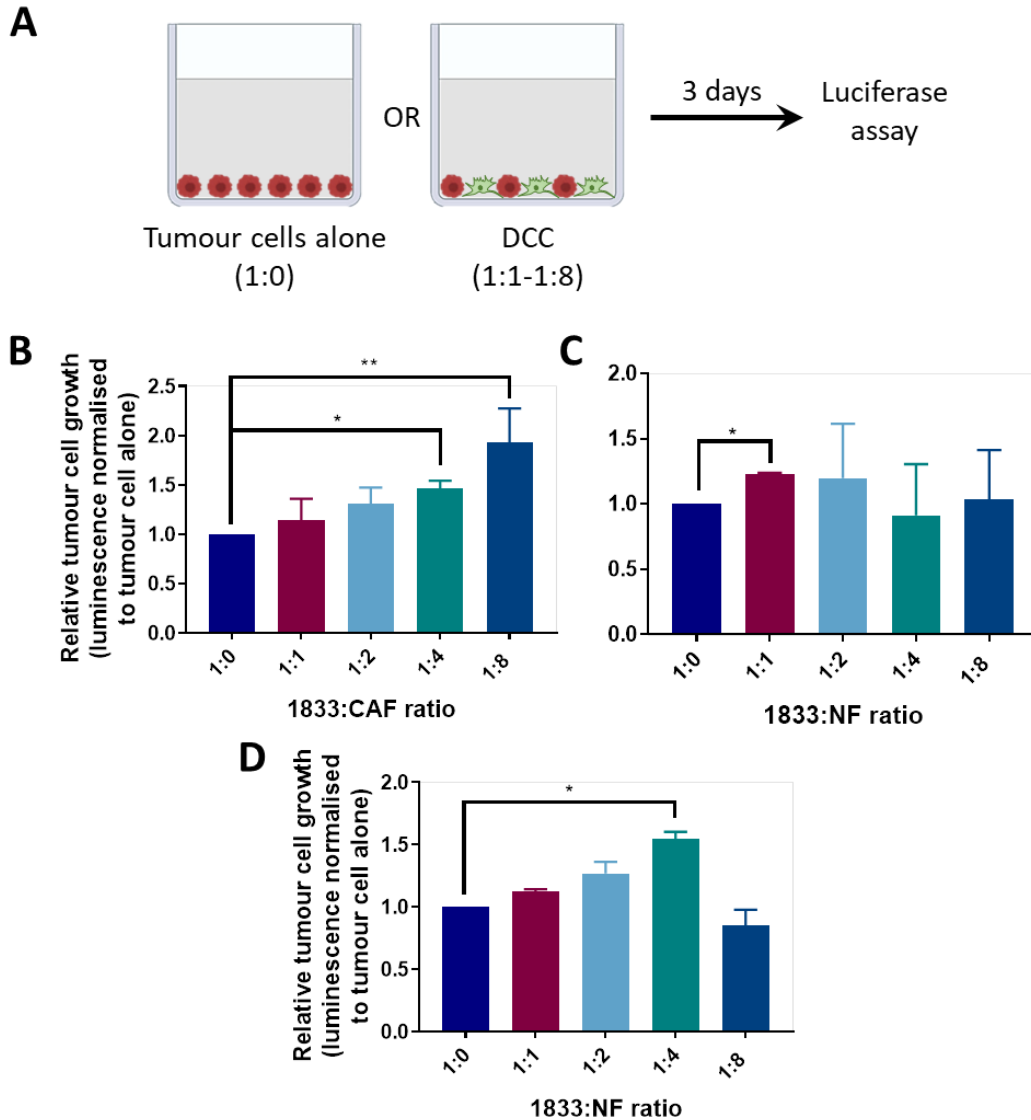


Figure 4.2.16: Direct contact between mesenchymal stromal cells and tumour cells promote tumour cell growth *in vitro*. **A)** Experimental design: proliferation assay of tumour cells alone, or in direct-co-culture with fibroblasts (red, tumour cells; green, fibroblast; DCC, direct co-culture). **B-D)** 1833 growth in culture with **B)** breast cancer-derived CAFs, and normal breast fibroblasts from **C)** patient 1 and **D)** patient 2. Cell growth was quantified by luciferase assay on day 3 of culture (NF, normal fibroblasts). Data shown are mean $\pm$ SEM (* p <0.05 ** p <0.01, one-way ANOVA, n =3-6).

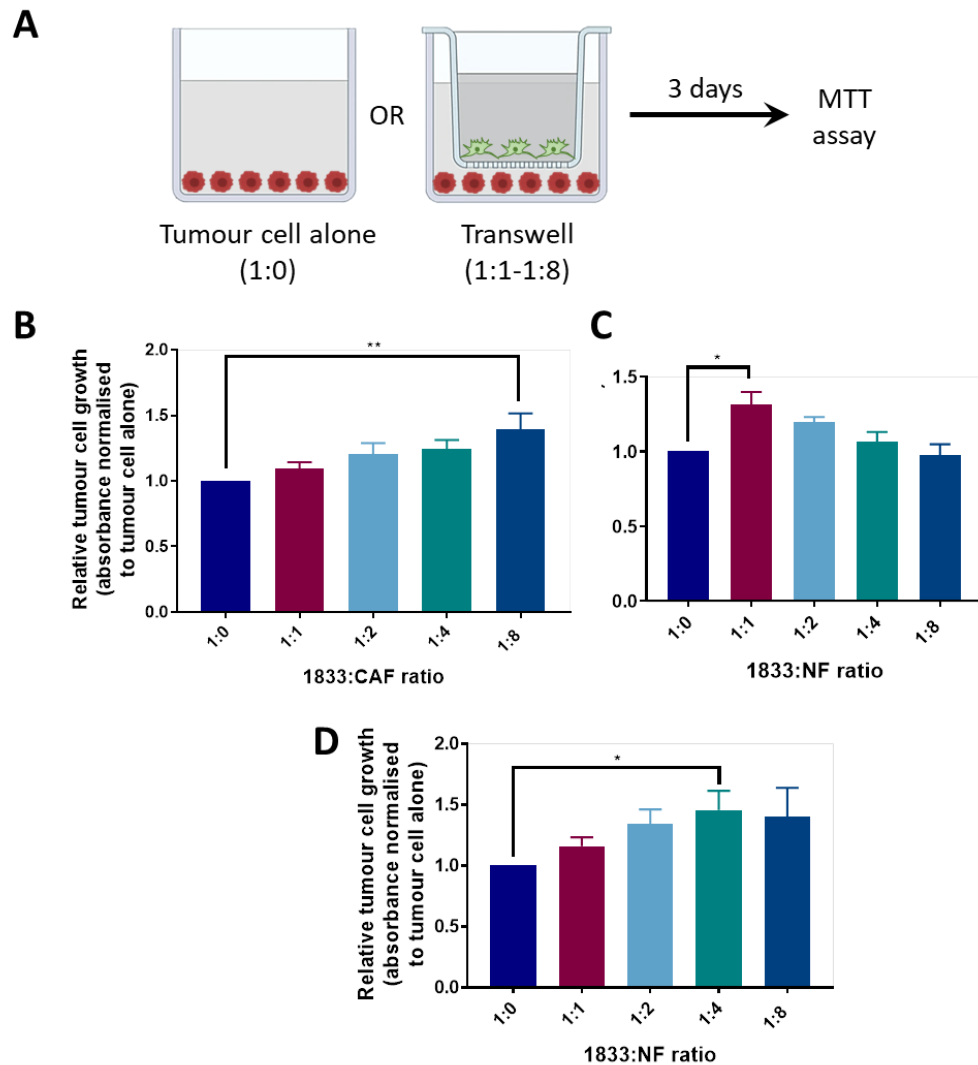


Figure 4.2.17: MSC-derived soluble factors promote the growth of tumour cells. A) Experimental design: proliferation assay of tumour cells alone, or with MSCs in a transwell. (red, tumour cells; green, fibroblast). **B-D)** 1833 growth in culture with **B)** breast cancer-derived CAFs, and normal breast fibroblasts from **C)** patient 1 **and D)** patient 2. Cell growth was quantified by MTT assay on day 3 of culture (NF, normal fibroblasts). Data shown are mean $\pm$ SEM (* p <0.05 ** p <0.01, one-way ANOVA, n =3-6).

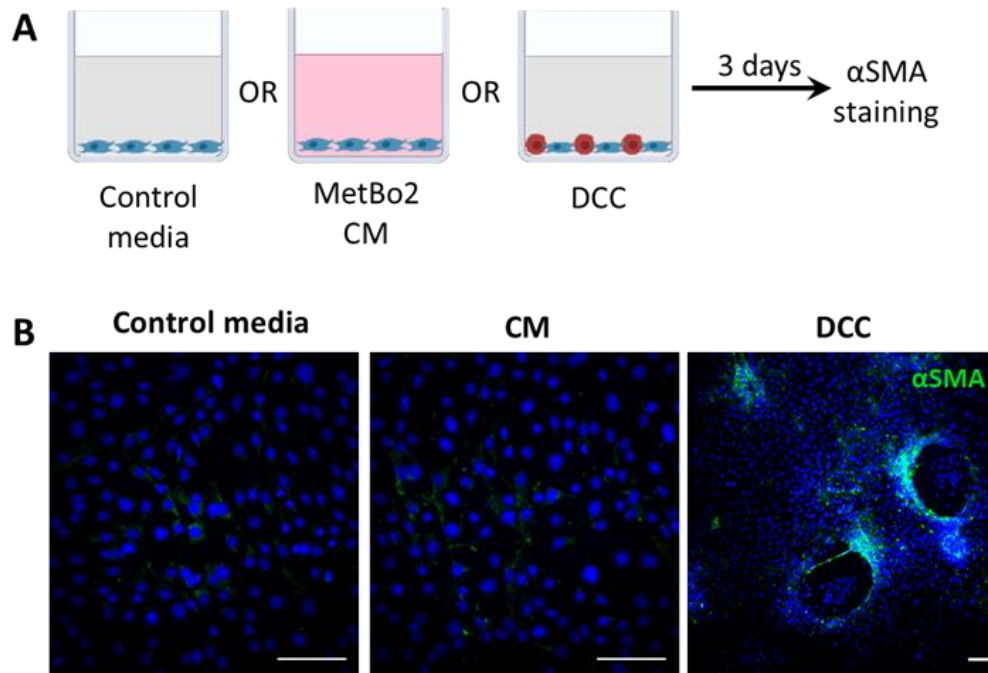


Figure 4.2.18: Direct contact between MSCs and tumour cells induces α SMA expression in MSCs. **A)** Experimental design: differentiation assay of MSCs in control media, MetBo2-derived conditioned media or direct co-culture with MetBo2 cells (blue, MSCs; red, MetBo2; DCC, direct co-culture). **B)** Immunofluorescence images of MSCs cultured in control media (left), MetBo2-derived CM (centre) and direct co-culture (DCC) with MSCs (right) stained for α SMA. Scale bar: 100 μ m. Representative images of 3 separate experiments performed in triplicate are shown.

4.2.3 MSC-macrophage cooperation in bone metastatic growth

Tumour-associated macrophages also contribute to tumour growth. Previous data in the lab demonstrates that a distinct population of TAMs in bone metastasis plays a significant role in growth and is attenuated after depletion of these TAMs results in ablation of tumour growth (Ma *et al.* submitted). Due to the interactions between macrophages and MSCs in normal bone^{171,283} and the previous MSC-mediated polarisation of TAMs observed in other cancer models, we hypothesise that a similar cooperation between TAMs and MSCs may exist in bone metastasis. To test this, we first performed a macrophage polarisation assay using macrophages and MSCs isolated from the bones of FVB/N mice (figure 4.2.19a). Macrophages were cultured either alone, in direct co-culture with MSCs or in conditioned media derived from MSCs. Whilst the majority of macrophages cultured alone exhibited a round or small spindle-like shape after 48 hours in culture, the macrophages cultured with MSCs or

MSC-derived conditioned media displayed a more mesenchymal phenotype with more and longer filopodia projections (figure 4.2.19b).

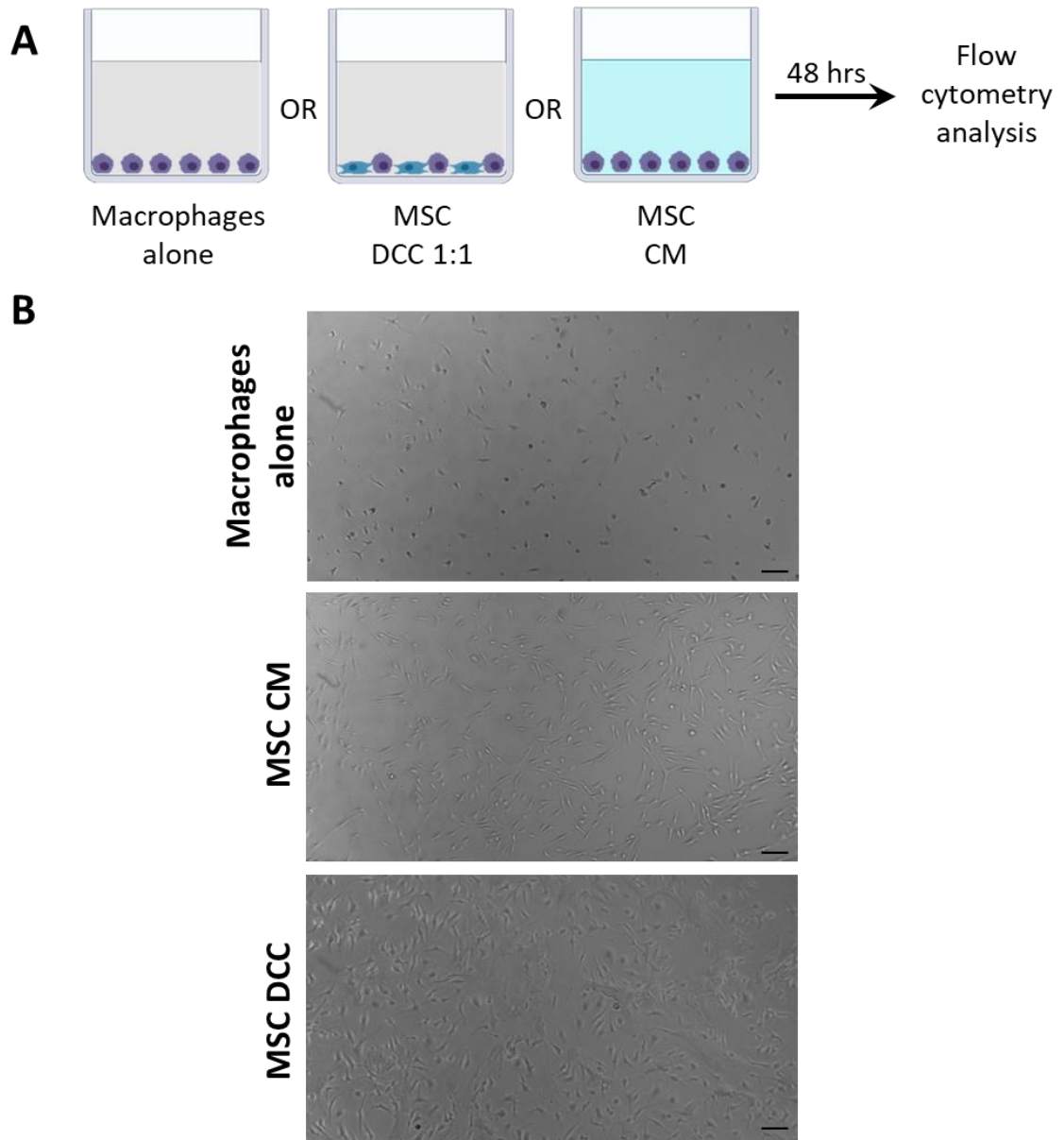


Figure 4.2.19: Macrophages cultured with MSCs exhibit more spindle-like and mesenchymal-like morphology than macrophages cultured alone. A) Experimental design: Polarisation assay of bone marrow-derived macrophages cultured alone or in direct-co-culture with bone marrow-derived MSCs (purple, macrophages; blue, MSCs; DCC, direct co-culture). **B)** Bright field images of macrophages cultured alone (top), in MSC-derived CM (middle) and with MSCs (bottom). Images are representative of 3 experiments (CM, conditioned media; DCC, direct co-culture). Scale bar: 100µm.

We next analysed the expression of tumour-associated markers by the macrophages using flow cytometry (figure 4.2.20). Macrophages were gated as CD45⁺ F4/80⁺ cells to eliminate MSCs in the co-culture.

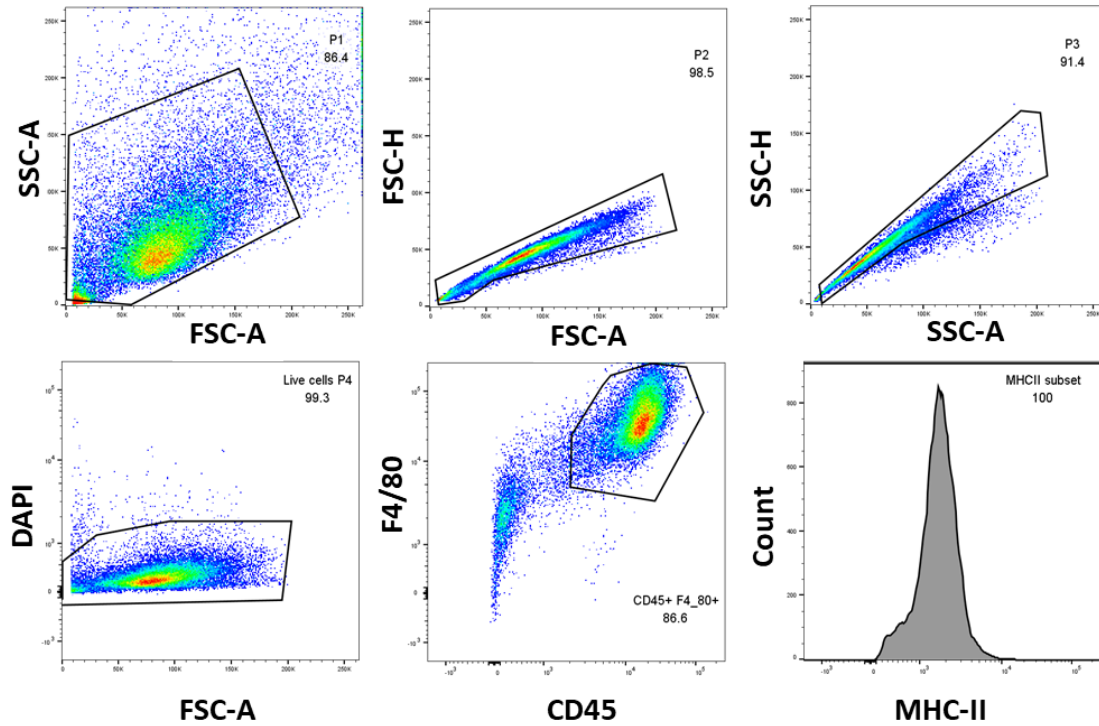


Figure 4.2.20: Representative gating strategy of the tumour-associated markers in macrophages.

There is a significant increase in the expression of PD1 and IL4R in macrophages during direct co-culture with MSCs, whilst there is no effect of MSC-derived conditioned media (figure 4.2.21a, figure 4.2.21b). The expression of CD204 also trends towards a significant increase direct co-culture with MSCs ($p=0.0586$), whereas the MSC-derived conditioned media ablated CD204 expression as the negative mean fluorescence intensity value suggests that there is no expression of CD204 (figure 4.2.21c). In contrast both the direct co-culture and MSC-derived conditioned media reduces the expression of PDL1, Tim4 and MHCII with significant trends observed in the MSC-derived conditioned media (figure 4.2.21d-f).

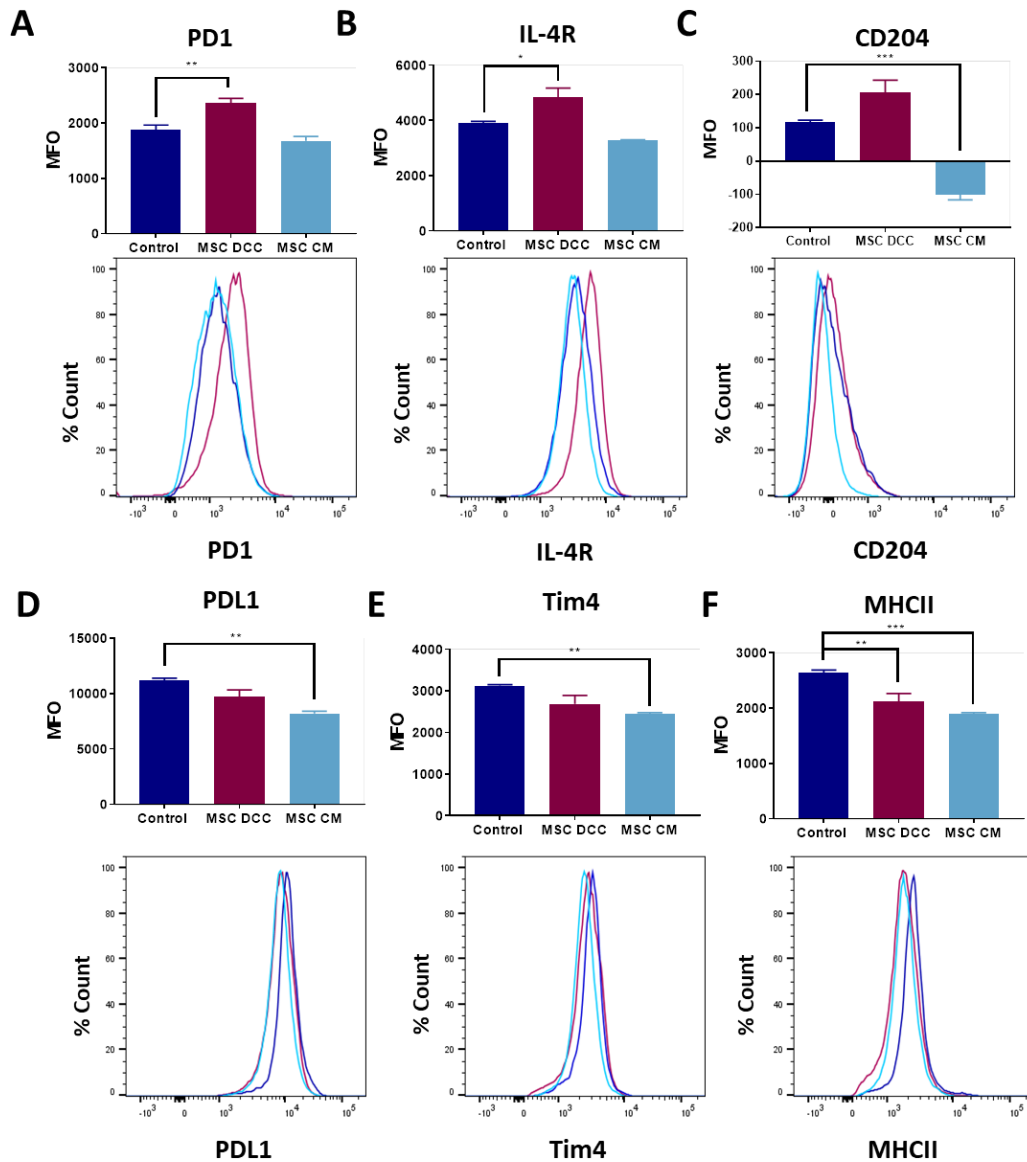


Figure 4.2.21: MSCs induce expression of macrophage markers associated with breast cancer bone metastasis. Quantification and representative flow cytometry histograms of **A)** PD1, **B)** IL4R, **C)** CD204, **D)** PDL1, **E)** Tim4 and **F)** MHCII expression on macrophages culture alone (control), in direct co-culture with MSCs (MSC DCC) and in MSC-derived conditioned media (MSC-CM). Data shown are mean $\pm$ SEM (* p <0.05 ** p <0.01 *** p <0.005, one-way ANOVA, n =4).

We next investigated the role of MSCs in recruitment of macrophages using *in vitro* migration assays. MSCs were seeded in 24 wells and allowed to grow to 80% confluence. The media was replaced with fresh media 24 hours before the migration assay. Fresh media only was also placed in the control wells. Macrophages were seeded in 8.0 μ m-pore transwell inserts at a concentration of 10,000 cells/transwell and added each well in the plate. Cells were left to migrate for 12 hours in the incubator

after which migrated cells were visualised using crystal violet staining (figure 4.2.22a). Quantification demonstrates that there is an increased number of migrated macrophages towards MSCs compared to media-only controls (figure 4.2.22b, figure 4.2.22c). Together, this preliminary *in vitro* data demonstrates that MSCs may contribute to the recruitment of macrophages to the bone metastasis and polarisation to a TAM-like phenotype.

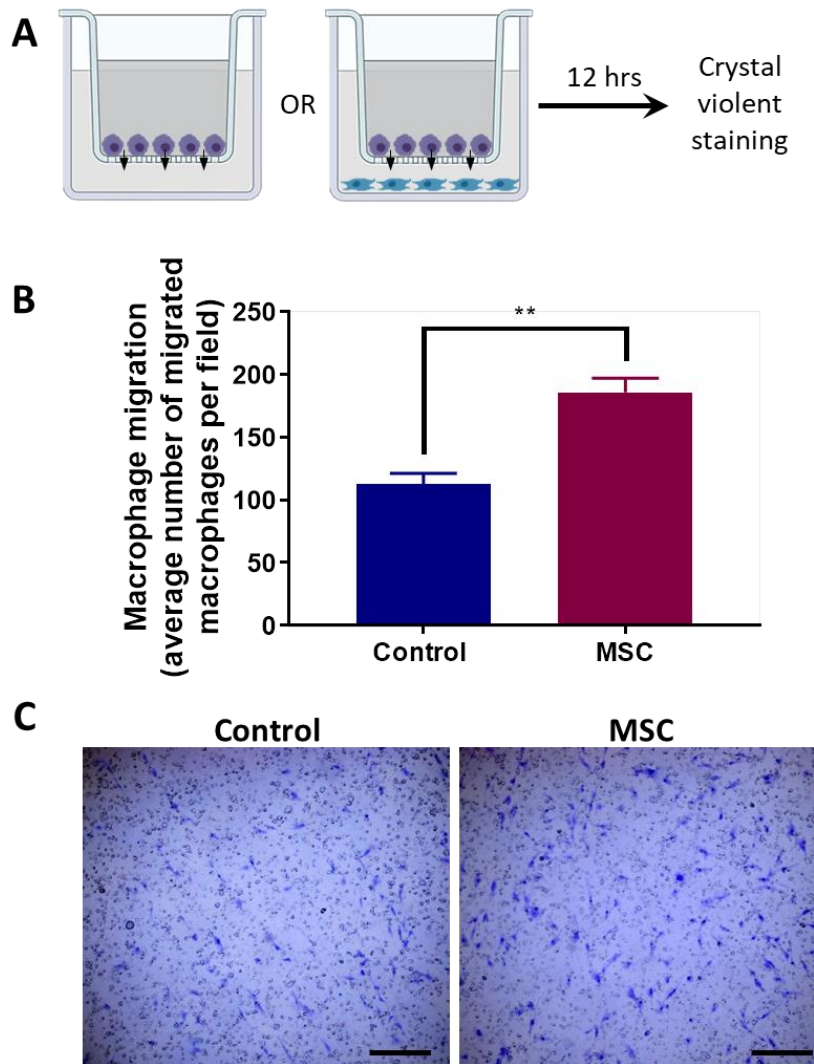


Figure 4.2.22: MSCs promote macrophage migration *in vitro*. **A)** Experimental design: migration assay of macrophages towards control media or bone marrow-derived MSCs (purple, macrophages; blue, MSCs). **B)** Quantification of macrophage migration across transwells towards control media or bone marrow-derived MSCs. Data shown are mean $\pm$ SEM (n=3). **C)** Representative images of macrophages in the transwells migrated towards control media (left) and MSCs (right). Scale bar: 200 μ m.

4.2.4 Role of osteoblasts in bone metastatic growth

Osteoblasts play a vital role in the 'vicious cycle' of bone metastatic growth. SP7, also known as osterix, is a transcription factor regulating osteoblast differentiation by activating genes during differentiation of preosteoblasts to mature osteoblasts. We therefore investigated the role of osteoblasts in breast cancer bone metastasis using SP7-GFP-Cre-iDTR nude mice. These mice were generated by the mating of B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J, C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J, Foxn1nu/Crl. The B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J mice use a tetracycline-off mechanism containing a Cre-mediated recombination under control of doxycycline (figure 4.2.23a). The absence of doxycycline after the removal from specialised diet induces expression of the eGFP-Cre fusion protein under the control of the SP7-promoter. The mating of these mice with C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J mice containing the floxed iDTR gene induces the expression of iDTR under the SP7⁺ osteogenic lineage (figure 4.2.23b). Subsequent administration of diphtheria toxin results in ablation of the SP7⁺ osteoblast lineage.

We established bone metastasis in these mice via intrailiac injection of MetBo2 cells. After tumours were detected, the mice were treated with DT or mutated Glu⁵²DT daily to induce SP7⁺ osteoblast depletion (figure 4.2.23c). Whole mount staining of bones collected from control and depleted mice was used to confirm the efficiency of depletion on day 9 of depletion. In non-depleted controls, SP7⁺ osteoblasts were located within the tumour regions, particularly around the edge of the tumour, whereas the osteoblasts were only located within the endosteal niche and epiphysis region of the normal bone (figure 4.2.24). As SP7 is an important transcriptional regulator of the osteoblastic differentiation of MSCs and therefore a marker for early osteoblasts, or preosteoblasts, this increase in SP7⁺ osteoblasts within the tumour region may indicate an enhanced differentiation of MSCs within the bone metastasis. In comparison to non-depleted controls, SP7⁺ osteoblasts were absent in depleted bones in the tumour region, epiphysis region and endosteal niche (figure 4.2.24). Whilst the bone metastatic regions exhibit increased SP7⁺ osteoblasts compared to the normal marrow, the depletion of SP7⁺ osteoblasts does not significantly affect tumour growth with a similar growth rate observed in both groups of mice (figure 4.2.25). However, there is a slight increase in tumour growth in the depleted group, albeit insignificant.

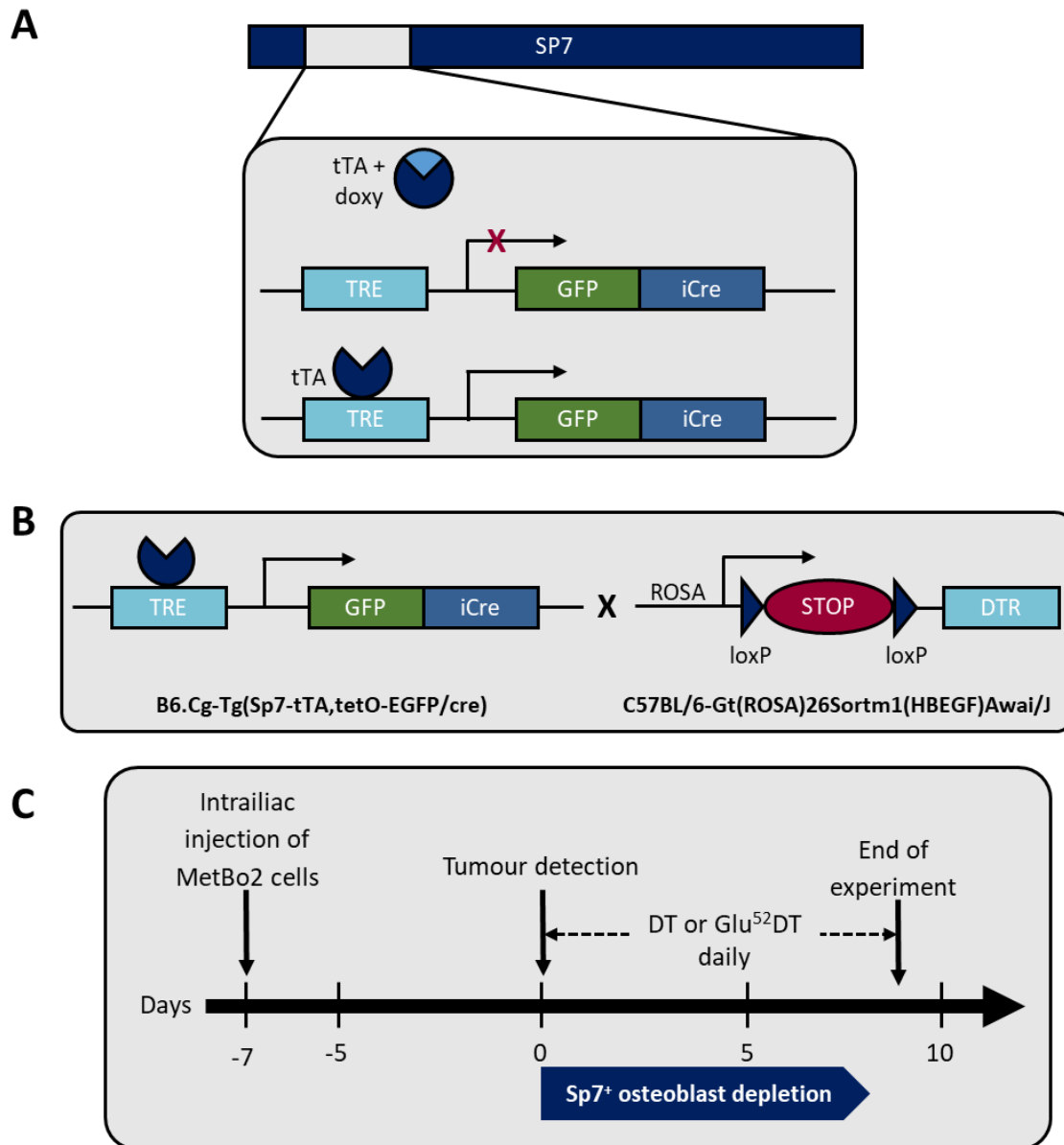


Figure 4.2.23: Effect of SP7⁺ osteoblast depletion on bone metastatic growth. A) Illustration of the tetracycline- controlled transcriptional system that regulates GFP and Cre expression in the B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre) mice (TRE, tetracycline response element; tTA, tet-activators; Doxy, doxycycline). **B)** Schematic diagram illustrating the mouse crosses and the genetic details of the strains used to produce the SP7-GFP-iCre-iDTR mouse model. **C)** Experimental design: experimental bone metastasis assay in SP7-GFP-Cre-iDTR mice. Administration of DT induced SP7⁺ osteoblast depletion, whilst control mice received Glu⁵²DT (DT, diphtheria toxin).

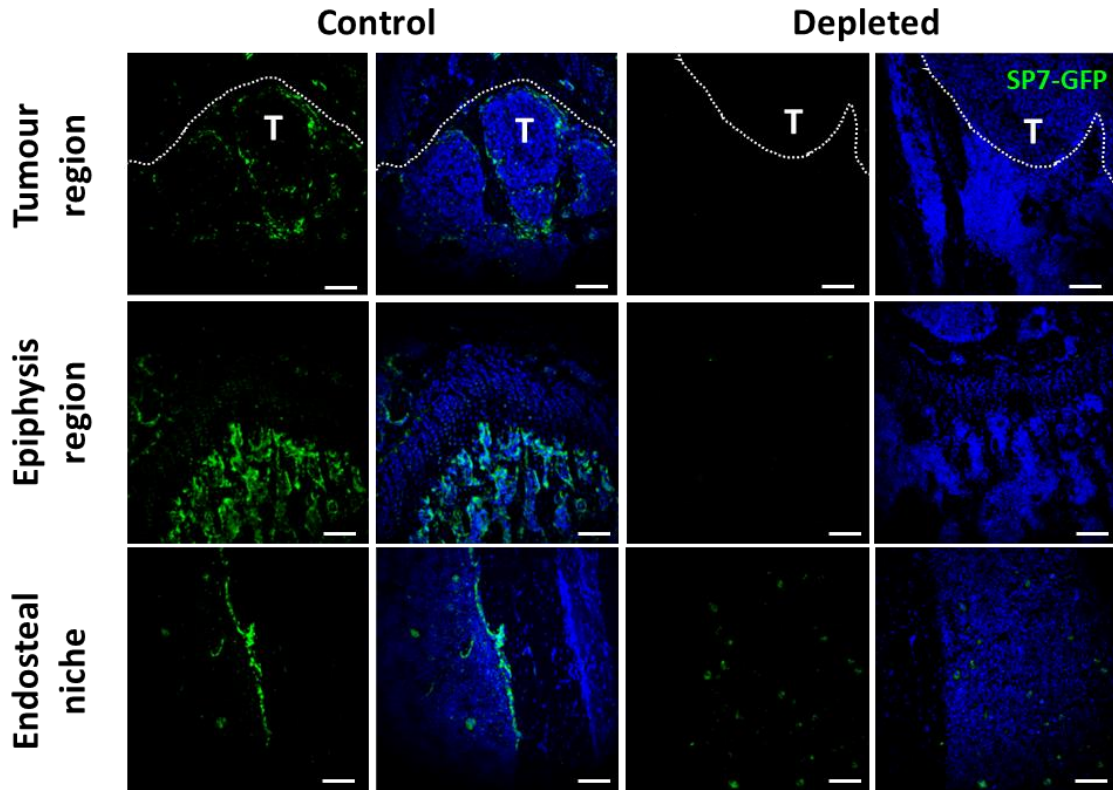


Figure 4.2.24: SP7⁺ osteoblast depletion is highly efficient in SP7-GFP-Cre-iDTR mice. Whole mount fluorescent images (maximum projection) of the tumour region (top), growth plate (middle) and endosteal niche (bottom) non-depleted control bones and SP7⁺ osteoblast depleted bones stained for SP7-GFP (left) and merged with DAPI (right). Representative images from 3 mice are shown. T, tumour area; scale bar: 100µm.

To further support these findings, we analysed the effect of osteoblasts on tumour growth using *in vitro* proliferation assays. MetBo2 cells were cultured alone or in direct co-culture with osteoblasts derived from murine bone marrow at a MetBo2:osteoblast ratio of 1:1-1:8. After 3 days, a luciferase assay was used to quantify the number of tumour cells (figure 4.2.2ba). The addition of osteoblasts to the culture of 1833 has no significant effect on 1833 growth, and reduces tumour growth at the ratio of 1:8 (figure 4.2.26b). This is an analogous with effects as observed *in vivo*.

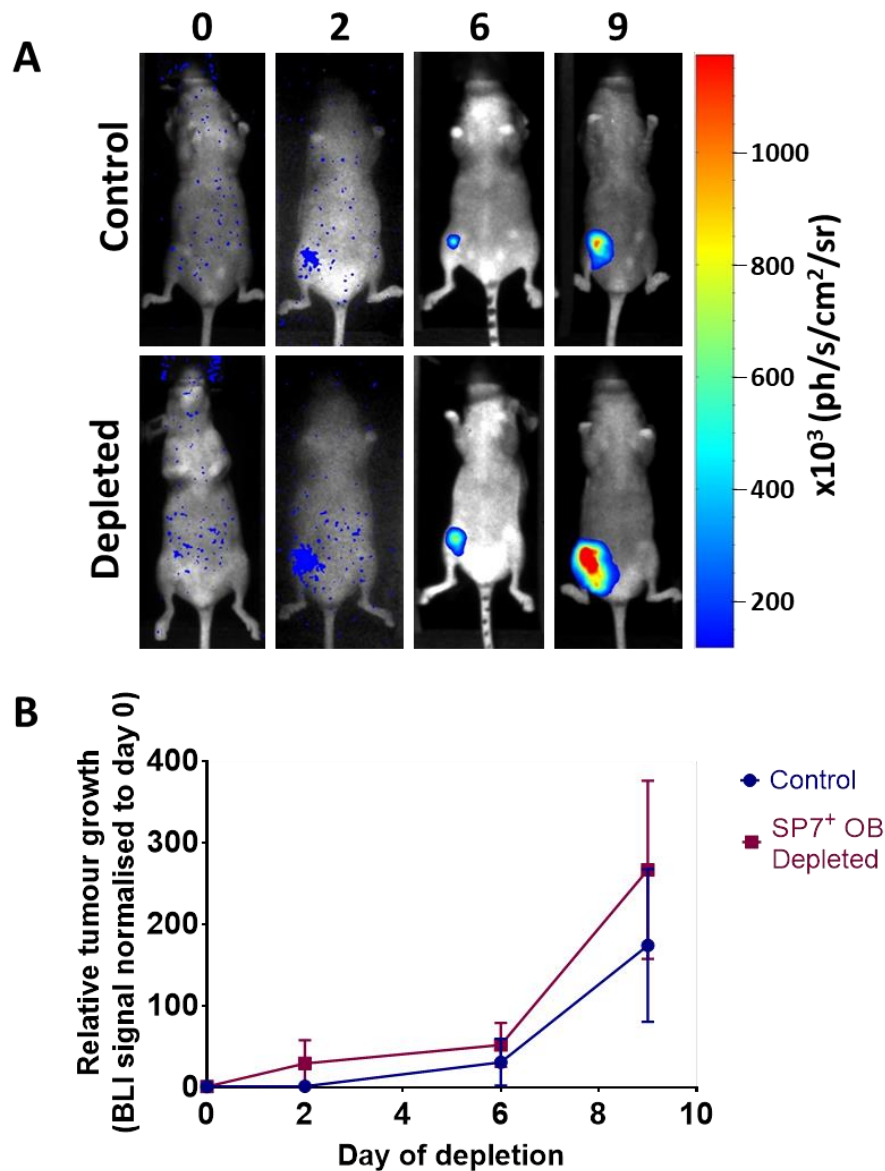


Figure 4.2.25: SP7⁺ osteoblast depletion has no significant effect on bone metastatic growth. A) Representative images of the BLI signal throughout SP7⁺ osteoblast depletion. **B)** Quantification of bone metastatic growth in control and SP7⁺ osteoblast depleted mice. Growth was monitored using bioluminescent imaging on day 0, 2, 6 and 9 of depletion (OB, osteoblast). Data shown are mean $\pm$ SEM (n=3-4).

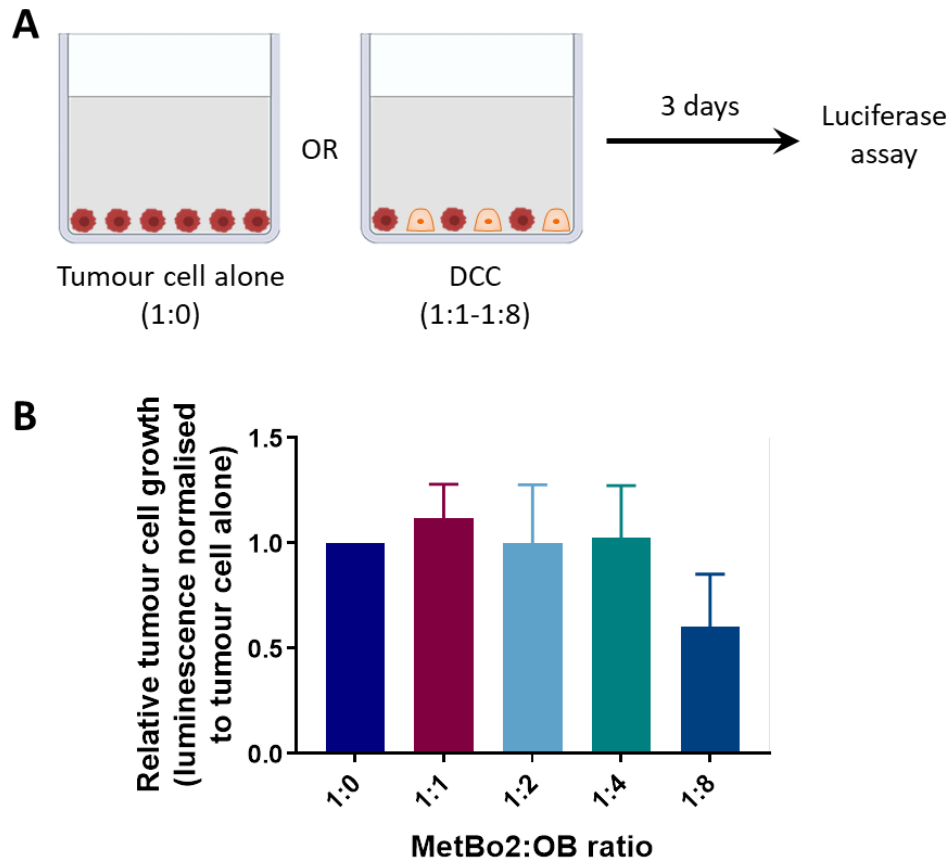


Figure 4.2.26: Osteoblasts do not affect tumour cell growth *in vitro*. **A)** Experimental design: proliferation assay of tumour cells alone, or in direct-co-culture with osteoblasts (red, tumour cells; orange, osteoblasts; DCC, direct co-culture). **B)** MetBo2 growth in culture alone or with osteoblasts quantified by luciferase assay on day 3 of culture. Data shown are mean±SEM (n=4).

4.3 Discussion

4.3.1 Effect of mesenchymal stem cells on breast cancer bone metastatic growth

Here, we demonstrate that Nestin⁺ MSCs play a vital role in the growth of breast cancer bone metastasis. This population of cells is recruited to the tumour region where they promote survival and growth of the tumour cells. The depletion of nestin⁺ MSCs in bone metastasis significantly reduced growth compared to non-depleted controls, resulting in near elimination of bone metastasis. The nestin⁺ MSCs are a vital component of the HSC niche⁷⁸, which was previously identified to provide a niche for survival of tumour cells within bone metastasis^{59,91}. In an analogous manner, this increased infiltration of nestin⁺ MSCs within the bone metastatic regions may therefore promote the growth of the tumour cells via the expansion of a HSC niche-like environment. We plan to confirm these findings in a human model of breast cancer bone metastasis using the 1833 cells. It would be valuable to investigate the effect of injection of MSCs into tumour-bearing mice as we expect they would be recruited to the bone metastasis where they would enhance growth.

The role of nestin⁺ MSCs is supported by previous studies that demonstrate a correlation between nestin expression and poor prognosis in breast cancer as well as other cancer models. However, the cell source of the nestin is often not specified or is expressed by other cells including cancer cells^{284–287}. The use of the Nestin-cre^{ERT2}/iDTR/GFP/nude mice confirms that the nestin is expressed by the host cells within the TME of the bone metastasis, rather than tumour cells, which have been shown to express nestin and play a role in tumour progression²⁸⁴. It also permits the specific depletion of the nestin⁺ MSCs, and whilst the tumour region exhibited efficient depletion of nestin⁺ MSCs, it is unclear why the MSCs remained in the normal region. One possibility is that the nestin promoter is more active within the bone metastatic regions compared to normal bone, which would coincide with the increased nestin area observed in bone metastasis compared to normal regions and support enhanced expression of nestin due to the bone metastasis. The increased activation of the nestin promoter would also result in increased depletion efficiency as the expression of diphtheria toxin receptor (DTR) is under the control of the nestin promoter and thus cells would exhibit augmented expression of the DTR on the cell surface. One drawback of this experimental model is the limited depletion duration. As MSCs are

important cells in homeostasis, such as HSC maintenance and bone turnover, as well as the inflammatory response during wound healing, repair and regeneration^{116,288}, the depletion of these cells in the mice caused adverse effects, including significant weight loss. This restricted the depletion duration to approximately 7 days after which the mice had to be sacrificed. It would be valuable to determine whether longer depletion duration would completely eliminate the bone metastasis.

In addition to the nestin⁺ MSCs, we further characterised the mesenchymal compartment of breast cancer bone metastasis using CD146 staining. CD146, also known as melanoma cell adhesion molecule (MCAM), is another MSC marker and has been associated with those at the vascular location in both normal tissues and the TME^{35,38,289–295}. CD146 is a membrane protein belonging to the immunoglobulin superfamily and functions as a receptor for several ligands including laminin alpha 4, galecin-1 and S100A8/9²⁹⁶. It is involved in the adhesion and migration of cells, and whilst this adhesive strength is relatively weak under normal physiological conditions in adult tissues, CD146 expression is upregulated and provides cell-cell interactions to promote cell proliferation, adhesion and migration during pathological conditions, including cancer and inflammation where CD146 binding permits invasion of the surrounding ECM²⁹⁷. Within breast cancer bone metastasis, we show an increased abundance of CD146⁺ cells in the tumour region compared to control bones. Whilst CD146 expression in tumour cells has been reported²⁹⁸, this was not observed in bone metastasis with an stromal pattern observed. Furthermore, we demonstrate that the abundance of CD146⁺ MSCs is significantly correlated with the number of proliferating cells in bone metastasis. This association supports a role of CD146⁺ cells in promoting bone metastatic growth. Notably, this is not observed in mammary fat tumours composed of the same tumour cells, suggestive that the effect is specific to the TME within the bone. CD146 expression is associated with poor prognosis and metastasis in breast cancer²⁹⁹ as well as other cancer models^{298,300,301}, although the source of CD146 expression is not identified in some models. The role of CD146⁺ MSCs in breast cancer bone metastasis may be determined using CD146-deficient mice, siRNA silencing or neutralising antibodies to inhibit CD146 function in future studies^{293,294}.

CD146 also promotes angiogenesis via several functions. Firstly, it acts as a co-receptor for pro-angiogenic factors including VEGFR2 where extracellular binding of CD146 to VEGFR2 on endothelial cells promotes VEGF-A-induced phosphorylation of VEGFR2 and subsequent intracellular signalling important for angiogenesis³⁰². It also

promotes vessel integrity by enhancing recruitment of pericytes to the blood vessel often through the interactions with PDGFR β ^{293,294}. As CD146 is associated with MSCs, or pericytes, surrounding the blood vessels, further characterisation of the CD146⁺ cells is also required to determine the location of expression throughout the TME in relation to the blood vessels. The increased abundance of CD146⁺ cells may be due to neoangiogenesis of blood vessels, which are surrounded by vascular MSCs, rather than an increase of disseminated CD146⁺ MSCs throughout the TME. Co-staining of the blood vessels using CD31 or endomucin should therefore also be performed. It is also important to determine whether there is overlap between the CD146⁺ and nestin⁺ MSCs, or whether they exist as distinct populations. Previous reports demonstrate that a fraction of Nestin⁺ MSCs also express CD146 as well as PDGFR α ³⁰³. Co-staining of CD146 and nestin-GFP in metastatic bones collected from Nestin-cre^{ERT2}/iDTR/GFP/nude mice will confirm this. The identification of mesenchymal cells that co-express CD146 and NG2 defined as pericytes³⁵, and the involvement of CD146 in the recruitment of PDGFR β ⁺ pericytes^{293,294} also prompts further characterisation of these cells. Staining should also be performed to identify NG2⁺ and PDGFR β ⁺ cells in bone metastasis, which is also significantly associated with a poor prognosis and recurrence in breast cancer³⁰⁴.

We further show using *in vitro* co-culture experiments that bone marrow-derived MSCs promote growth of bone metastatic breast cancer cells in both the murine and human model. These effects were only observed in direct co-culture where growth of tumour cells was correlated with the number of MSCs in the co-culture. In contrast, there was no effect of MSCs on tumour growth in the transwell assays. This suggests that the tumour-promoting effects of nestin⁺ MSCs were dependent on direct contact between the MSCs and the tumour cells, whilst MSC-derived soluble factors have no or little effect on growth. It may also be useful to repeat a similar assay by culturing tumour cells in a decellularised or acellular matrix produced by the MSCs as this would give insight into the role of MSC-derived extracellular matrix proteins in the promoting tumour growth. The *in vitro* proliferation assay also confirms that the MSC-induced growth is at least partially mediated by factors that act directly on the tumour cells to promote growth.

Future directions should aim to identify potential mechanisms by which mesenchymal cells exert their pro-tumorigenic effects. To identify molecules upregulated in the bone metastatic MSCs, RNA-sequencing, cytokine arrays and/or qPCR will be performed on

nestin-GFP MSCs sorted from normal and metastatic bones. Similarly, gene expression analysis of MSCs exposed to breast cancer cells in culture compared to naïve MSCs may offer insight into the molecular mechanisms involved in both the activated phenotype of the MSCs and their tumour-promoting effects. Whilst our *in vitro* data suggests that the MSC-tumour interaction involves direct contact, alternative mechanisms may be observed *in vivo*. The expression of potential molecules in the bone metastasis can be identified using immunostaining or flow cytometry, and can then be functionally tested using siRNA knockdown or inhibitors to confirm a functional role.

There is now abundant evidence to support that mesenchymal cells promote tumour growth through the secretion of factors and that directly act on the tumour cells to promote proliferation. This has been demonstrated in several cancer models, including breast, lung, prostate and melanoma as summarised in table 4.3.1.

Mesenchymal stromal cell source	Cancer type	Growth factor
MSC	Breast	CCL5 ³⁹
	Breast	IL6 ⁴⁰
	Breast	CXCL7 ⁴¹
	Breast	CXCL12–CXCR4 ^{42,43}
	Breast	Collagen-DDR2 ⁴⁴
	Prostate	TGFβ ⁴⁵
	Neuroblastoma, osteosarcoma, colorectal	IL6 ^{47–50}
Senescent MSCs	Colorectal	Galectin 3 ⁴⁶
MSC-derived CAF	Ovarian	IL6, HGF, EGF ⁵¹
	Gastric	IL6, Wnt, BMP ⁵²
	Liver	miR155 ⁵³
	Breast primary	CXCL12 ⁵⁴
CAF	Breast	CXCL12–CXCR4 ^{55,56}
	Breast	VEGF ⁵⁷
	Breast	HGF ⁵⁸
Osteoblast	Prostate bone metastasis	CCL2 ⁵⁹

Table 4.3.1: Factors implicated in mesenchymal-induced growth. MSCs, CAFs and osteoblasts secrete factors that act on tumour cells to induce growth.

Within the bones of a multiple myeloma model, a complex paracrine loop involving MSCs promotes the growth of osteolytic lesions. MSCs secrete unidentified factors to promote the secretion of Wnt inhibitor, DKK1, from the myeloma, which inhibits the MSC differentiation to osteoblasts. Instead the undifferentiated MSCs express IL6 that promotes the growth of the myeloma cells, and was attenuated using neutralising IL6

antibody. In turn, the myeloma cells secreted more DKK1 therefore establishing a positive feedback loop, which could be disrupted by Wnt activator, 6-bromoindirubin-3-monoxime³⁰⁵. Mesenchymal cells are also a major source of CXCL12^{129,306}, which is one of the growth factors implicated in bone metastatic growth^{95,129}. CXCL12 was increased in bone metastatic prostate cancer but not in localised tumours or normal prostate tissue. CXCL12 stimulated prostate cancer growth *in vitro*, which was inhibited using neutralising antibody, suggesting that CXCL12 mediates growth at the metastatic site.³⁰⁷ Although stromal-derived CXCL12 is implicated in the growth of several cancers, it may also be involved in dormancy³⁰⁸. These functional differences may be due to the Src-status of the tumour cells, which mediates responses to CXCL12 produced by the bone microenvironment. CXCL12-CXCR4 interactions activate Akt signaling in the cancer cells to promote survival and resistance to TRAIL death signals, which is potentiated by Src. Src-depleted breast cancer cells exhibited attenuated growth within the bone, which was restored by Src expression. Furthermore, Src is associated with relapse in the bone, but not other organs, suggesting that it is necessary for the survival and outgrowth of latent bone metastasis³⁰⁹. Cells expressing high Src may be selected for in the primary tumour. CAFs secrete CXCL12 and IGF1 to select for hyperactive Src clones, which are highly metastatic to the bone. This means that once these cells reach the bone marrow they are selected to survive in the CXCL12 environment⁹⁵.

Mesenchymal stromal cells can also promote angiogenesis, which is important for both primary tumour and metastatic growth, particularly as the tumour size increases to ensure delivery of nutrients and oxygen³¹⁰. Angiogenesis involves the activation of the endothelial cells that migrate to form new blood vessels. MSCs secrete a variety of angiogenic factors during both wound healing and hypoxic stress, including FGF2, FGF7, IL1, IL6, IL8 placental growth factor, TGF β , TNF α , EGF, CXCL12, angiopoietin1, erythropoietin, and PDGF and VEGF³¹¹. In prostate cancer bone metastasis, tumour-derived PTHrP induces CCL2 from MSC-derived osteoblasts, which acts on the tumour cells to induce VEGF secretion and therefore promotes angiogenesis³¹². It was also shown in multiple myeloma that endothelial cells exhibited increased migration to the bone marrow of multiple myeloma patients compared to healthy bone marrow due to increased angiogenic mononuclear cells in the bone marrow of patients. Inhibition of CXCR7 on the angiogenic mononuclear cells reduced their recruitment to the multiple myeloma enriched bone marrow niche and subsequently decreased angiogenesis resulting in attenuated tumour growth^{95,313,314}.

Although further research into the effect of stromal cells on angiogenesis within bone metastasis is required, evidence from studies in primary cancer confirms a pro-angiogenic role of MSCs due to the secretion of angiogenic factors^{315–319}. As observed in several tumour models, including prostate and breast cancer, tumours established from co-injection of MSCs and tumour cells were larger and exhibited increased proliferation compared to those injected with tumour cells only. This was correlated with increased blood vessel density. Together with other functional experiments, this demonstrates that MSCs augmented tumour growth as a result of increased angiogenesis due to release of VEGF, TGF β and IL6 from the MSCs³²⁰. Breast CAFs also secrete CXCL12 to enhance angiogenesis by recruiting endothelial cells to the tumour³⁰⁶. In hepatocellular carcinoma, MSC-derived TGF β , but not VEGF, caused an increase in angiogenesis and correlated with increased microvessel density³²¹, whereas VEGF was important for angiogenesis in pancreatic carcinoma³²². Co-transplantation of colon cancer cells and MSCs to establish subcutaneous xenografts also caused increased angiogenesis and tumour growth^{323,324}. This was due to a paracrine network in which MSC-secreted IL6 stimulated endothelin-1 secretion from the tumour cells that activated Akt/Erk in endothelial cells resulting in enhanced³²⁴. Another MSC-derived factor involved in angiogenesis in colorectal cancer is IL8, which induces migration and tube formation by endothelial cells³²⁵. MSCs and MSC-derived CAFs can also promote angiogenesis in ovarian cancer via the secretion of hypoxia inducible factor (HIF)1 α , CXCL12 and VEGF^{326–328}. Furthermore, ovarian cancer cells stimulate the secretion of proangiogenic factors from MSCs, such as IL6, TGF β 1, VEGF and HGF, highlighting the importance of the bi-directional paracrine networks established between the tumour cells and stromal cells in inducing angiogenesis^{326,329,330}. As well as the direct effects on endothelial cells, including migration and tube formation, MSCs may also promote angiogenesis by recruiting M2 macrophages, which secrete angiogenic cytokines such as IL6, IL8 and VEGF³²⁶. Many of the factors implicated in angiogenesis and growth of primary tumours are upregulated in bone metastasis, including CXCL12, TGF β 1 and IL6³³¹, therefore may play a similar role in promoting bone metastatic growth.

To determine if Nestin⁺ MSCs contribute to angiogenesis within breast cancer bone metastasis, the blood vessel density in nestin-depleted bone metastasis may be compared with non-depleted controls. CD146⁺ MSCs may contribute to neoangiogenesis by differentiation into vascular smooth muscle cells. It has previously been demonstrated that CD146 expression was associated with differentiation towards

a vascular smooth muscle lineage, suggesting CD146⁺ MSCs give rise to these cells²⁹⁰. These studies were performed within normal bone marrow, and Patenaude *et al.* previously demonstrated that CD146⁺ CD45⁻ CD31^{-low} pericyte cells, were a distinct population from the vascular smooth muscle cells in the TME²⁹⁵. Other MSCs may also contribute to angiogenesis via differentiation to endothelial cells. It has been shown that co-culture of MSCs with B16 melanoma cells or lewis lung carcinoma cells induced the upregulation of endothelial markers, including von Willebrand factor (VWF) and CD31³³². Treatment of MSCs with VWF and fibroblast growth factor (FGF) also increased endothelial cell markers and enhanced ability to form capillary like structures^{333,334}. However, this is controversial and may be an artefact of *in vitro* cultures as it has not been observed *in vivo*.

4.3.2 Origin of MSCs within bone metastasis

We next sought to identify the origin of MSCs recruited to the bone metastasis and demonstrate that soluble factors from the tumour cells induce both migration and proliferation. Mesenchymal stromal cells within the TME evolve from several different mechanisms, including recruitment from other tissues and activation or proliferation of resident cells (figure 4.3.1). Other sources of mesenchymal stromal cells, such as the EMT or differentiation from other cell types, for example smooth muscle and endothelial cells, will not be discussed here due to a lack of reliable evidence of this process *in vivo*³³⁵.

4.3.2.1 Recruitment

MSCs are recruited to tumours by the secretion of tumour-derived chemoattractants¹¹¹⁻¹¹³. Labelled MSCs injected into tumour bearing mice were detected within the tumours, where they persist for longer than in normal tissue^{111-113,336}. MSCs also display increased migration towards cancerous cells compared to the normal tissue counterpart, as demonstrated in prostate cancer where an increased abundance of injected MSCs were recruited to cancerous prostate tissue compared to normal prostate³³⁷. Interestingly, MSCs also display a greater tropism towards tumours compared to inflammatory tissues undergoing wound responses, demonstrating the effects are specifically due to the tumour, rather than the inflammatory-like environment¹¹². Whilst this tropism towards tumours has been demonstrated in several cancer models, most previous research is performed in models of primary tumours and

research to the secondary sites is limited to breast cancer lung and liver metastasis^{112,338}.

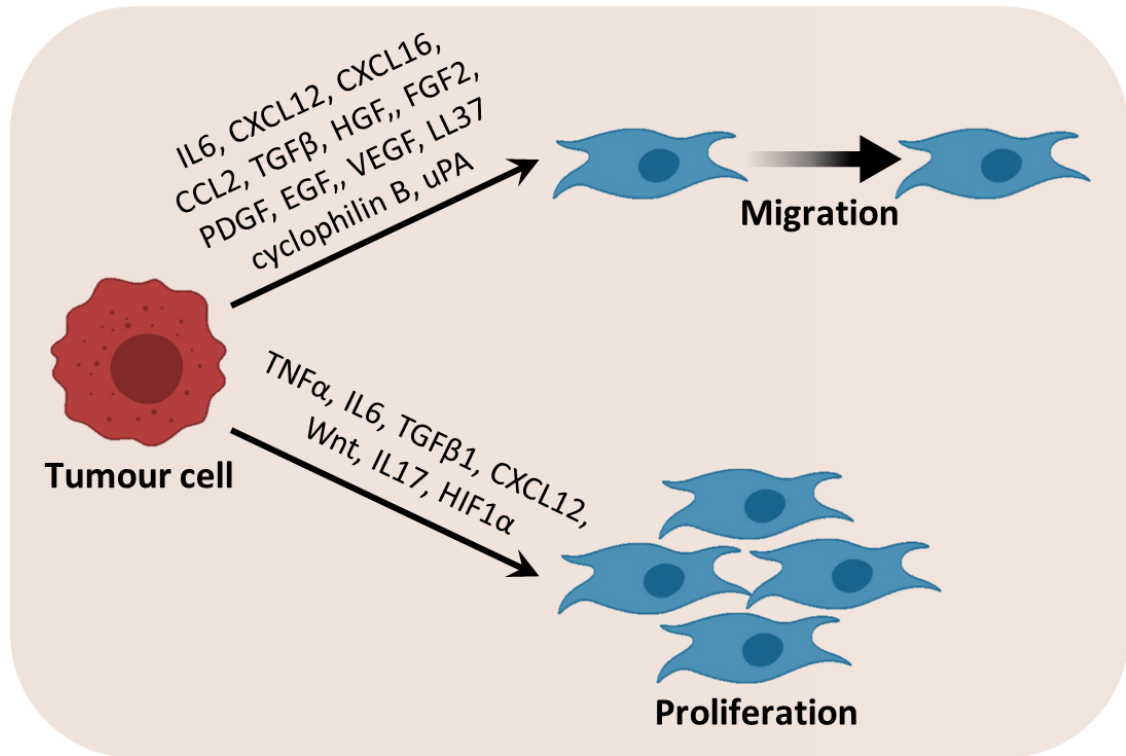


Figure 4.3.1: Origins of MSCs in the TME. Tumour cells induce migration and proliferation via the secretion of chemoattractants and growth factors.

Here, we demonstrated the bone metastatic breast cancer cells induce the migration of MSCs via the secretion of soluble factors. This suggests that MSCs may be recruited to bone metastasis via the secretion of tumour cell-derived chemoattractants and account for the MSC infiltration observed *in vivo*. The recruitment of MSCs to bone metastasis requires confirmation *in vivo* by performing adoptive transfer experiments.

Fluorescently-labelled MSCs can be injected into tumour bearing mice and can be monitored and tracked using immunofluorescence staining and intravital imaging of bone metastasis. To optimise this, MSCs were isolated from mice, including mTmG and Nestin-GFP⁺ mice, cultured *in vitro* and sorted to select fluorescent cells. MSCs were then injected into mice bearing bone metastasis, which were established via intracardiac injection of tumour cells. MSCs were injected via intrailiac injection to deliver them directly to the bone region. Unfortunately, injection of MSCs via this route was often lethal to the mice, possibly due to embolism formed of the MSCs within the

arteries. Despite alterations to the trypsinisation steps and MSC storage prior to injection, these issues persisted. Other injection routes, including intracaudal injection and intravenous injection have previously been used to inject MSCs successfully and are being investigated. Consistent with our *in vitro* data, we hypothesise that the injected MSCs will be found located within the bone metastasis due to recruitment mediated by tumour-derived chemoattractants. We will also inject MSCs into non-tumoral control mice, where we expect significantly less MSCs to be recruited to the normal bone.

Further research, including cytokine arrays of the tumour-derived conditioned media and identification of receptors expressed by the MSCs, may suggest potential molecular mechanisms, which can be tested functionally using molecular knockdown or inhibition experiments. Previous research may also offer insight into potential molecular mechanisms. Both bone and bone metastasis secrete cytokines such as VEGF^{339–341} and CXCL12⁹⁵ for which MSCs express the receptors^{342,343}, and therefore may serve as a potential molecular mechanism to induce MSC recruitment to bone metastasis.

The bone marrow and metastatic region also exhibit a hypoxic environment^{344–347}, which may induce MSCs recruitment to bone metastasis, as demonstrated in ischaemia models^{348,349}. Hypoxia is often a key feature of a growing tumour and has been shown to induce MSC migration via hypoxia-induced secretion of IL6 from breast cancer cells. Inhibition of IL6 using neutralizing antibodies reduced MSC migration towards breast cancer cells³⁵⁰. Previous studies also demonstrate using *in vitro* migration assays that MSCs migrate towards breast cancer cells due to a HIF α -dependent secretion of CXCL16, which interacts with CXCR6 on the MSCs. Breast cancer xenografts established from CXCL16-deficient tumour cells exhibit reduced MSC abundance and results in decreased circulatory tumour cells and metastasis to the lung³⁵¹. CXCL16 was also implicated in MSC recruitment to prostate cancer where prostate cancer-derived CXCL16 induces MSC migration via interaction with MSC-expressed CXCR6¹²⁹. Hypoxia-induced HGF may also mediate recruitment as demonstrated in a hind leg ischaemia model where hypoxia-induced HGF resulted in increased migration of MSCs due to upregulation of ERK/Akt signalling³⁴⁹. HGF is upregulated in the serum of breast cancer patients^{352,353}, and is associated with tumour formation and metastasis of breast^{354,355} and prostate cancer³⁵⁶. Furthermore, tumour cells-induced activation of HGF receptor, MET, in the bone marrow stromal cells and is associated with metastasis in a model of melanoma³⁵⁷. CCL2, also involved in inducing

MSC migration towards hypoxic environments³⁵⁸, also recruits MSCs towards breast cancer cells^{359,360}. Multiple myeloma, which locates to the bone, secretes CCL25, which induces MSC recruitment to tumour cells *in vitro* and tumours *in vivo*³⁶¹.

Several studies have demonstrated that MSCs are recruited to primary breast cancer via the secretion of tumour-derived chemoattractants therefore may play a similar role in breast cancer bone metastasis¹¹⁷. *In vitro* migration assays have been utilized to determine the molecular mechanisms mediating the interactions between breast cancer cells and mesenchymal cells, including CCL2, cyclophilin B, Hepatocyte-derived growth factor (HDGF), IL6, FGF2, VEGF and urokinase plasminogen activator (uPA). Inhibition of these molecules or their receptors using neutralizing antibodies or siRNA-silencing results in reduced MSC migration towards tumour cells or tumour cell-derived conditioned media *in vitro*^{359,360,362–364}. CXCL12 also recruits tumour-promoting MSCs to MCF10-derived breast cancers *in vivo* and inhibition of CXCL12 prevented recruitment of MSCs and lung metastasis³⁶⁵. The recruitment of labelled MSCs towards tumours after injection into tumour bearing mice was dependent on CXCL12/CXCR4 signalling in other cancer models, including subcutaneous xenografts of osteosarcoma³⁶⁶ and colorectal cancer³⁶⁷. Notably, bone metastasis are enriched with CXCL12⁵⁸, therefore may play a similar role in recruiting MSCs. Another molecule implicated in MSC recruitment is tumour-derived osteopontin, which induced the migration of MSCs to the breast cancer lung and liver metastasis in a xenograft model³³⁸ and may play a similar role in bone metastasis. Similarly, TGFβ1, which is upregulated in patients with metastasis, has also been implicated in MSC recruitment to gastric cancer³⁶⁸ as well as breast cancer where bone marrow-derived MSCs migrated to the breast cancer tumour, which was partially prevented by TGFβ inhibition. Moreover, the MSCs had an increased affinity towards aggressive and bone metastatic breast cancer cell lines (MDA-MB-231) compared to less aggressive and non-metastatic cells (MCF7) both *in vitro* and *in vivo*³⁶⁹.

Alternative tumour-derived factors contribute to MSC recruitment in other cancer models, including PDGF, EGF, VEGF, pro-inflammatory peptide LL-34, and angiogenic cytokines IL8, VEGF, neurotrophin-3, and TGFβ1^{93,163,322,370}. Similar signaling between the tumour cells and MSCs may contribute to MSC recruitment to breast cancer bone metastasis.

4.3.2.2 Proliferation

We also demonstrate that intratumoural MSCs exhibit increased proliferation within the bone metastasis compared to normal control MSCs. *In vitro* assays demonstrated that MetBo2-derived conditioned media promotes MSC proliferation, suggestive that the increased proliferation is at least partly promoted by tumour-derived soluble factors. Whilst the effect of cancer on the MSC proliferation is not well studied, our results are supported by several studies demonstrating that cancer cells induce proliferation of MSCs.

The cancer-mediated induction of MSC proliferation has previously been observed in several models of breast cancer. *In vitro* culture of human bone marrow-derived MSCs in conditioned media derived from TNBC resulted in increased numbers of MSCs. This was partially mediated by tumour-secreted IL6 and signal transducer and activator of transcription 3 (STAT3) signaling³⁷¹. Similarly, conditioned media collected from RCAS-Neu and RCAS-PyMT breast cancer cells increased the proliferation of bone marrow-derived MSCs, although the mechanism was not investigated³⁷². Studeny *et al.*³⁷³ demonstrated that bone marrow-derived MSCs display a proliferative phenotype in an *in vivo* murine model of lung metastatic melanoma. Melanoma A375SM-derived xenografts were established by subcutaneous injection of melanoma cells and fluorescently labelled MSCs. MSCs were also labelled with a lipophilic dye that decreases in intensity as the cells proliferate, which was observed in MSCs within the melanoma. This demonstrates a proliferative phenotype, and was confirmed using *in vivo* BrdU labeling was used to identify proliferating MSC³⁷³. Together, this provides direct *in vivo* evidence of MSC proliferation within melanoma tumours. In a model of gastric cancer, TGF β 1 induced proliferation of MSCs, which was inhibited by CXCR4 antagonist, indicating a complex molecular interaction between TGF β 1 and CXCL12-CXCR4 signaling³⁶⁸. Early studies also demonstrated that MSC proliferation is induced by TGF β 1 in a Smad3-dependent manner^{374,375}. The Wnt family also influence MSC proliferation with canonical Wnt3a inducing MSC proliferation³⁷⁶, whereas non-canonical Wnt5a suppresses proliferation³⁷⁷.

Although there are limited studies investigating the molecular interactions responsible for intratumoural MSC proliferation, the mechanisms involved in MSC proliferation in a non-tumoral context may provide insight. Huang *et al.*³⁷⁸ demonstrated that IL17 is an important factor for proliferation. They show that T cell-derived IL17 induces proliferation of human bone-marrow MSCs in a manner dependent on the generation of

ROS. IL17 activates both Rac1 GTPase and NADH oxidase 1, which produce reactive oxygen species (ROS) and subsequently stimulates MSC proliferation³⁷⁸. Omentin-1 also exerts pro-survival effects on bone marrow derived MSCs by promoting proliferation and inhibiting apoptosis via activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway³⁷⁹.

Bone marrow MSCs also exhibit an enhanced proliferative state under hypoxic conditions with a higher percentage of cells within the S-phase of the cell cycle^{380–383}. Senescence and apoptosis was also inhibited, whilst differentiation ability was increased. Kwon *et al.*³⁸³ also demonstrated that hypoxic MSCs exhibit an increased expression of HIF1 α in comparison to MSCs cultured under normal conditions^{381,383} and is supported by another study that implicated HIF1 α in the proliferation of human MSCs where crosstalk between the HIF1-SUMO-Notch pathways led to an increase in MSC proliferation. This effect was particularly prominent in hypoxic conditions when HIF1 α is stabilised³⁸⁴. MSCs exhibiting a proliferative phenotype induced by hypoxia also upregulate other factors including IGF1, FGF2, Nestin, and connexin43^{382,383}, which we or other studies have implicated in bone metastasis^{95,129}. Interestingly, migratory response to CXCL12 is also increased and may enhance recruitment to tumour regions^{366,367}. The MSC proliferation observed in bone metastasis may therefore be attributed to the hypoxic environment of bone metastasis^{344–347}.

4.3.2.3 Activation

Cancer-associated mesenchymal cells also display an activated phenotype compared to their counterparts from normal tissue. Exposure of MSCs to tumour cells and tumour-derived factors, such as TNF α , induces an 'activated' phenotype that exerts more pro-tumorigenic effects than 'naïve' cells^{367,380,385}. Many of these alterations include gene expression profile changes³⁶⁷ and secretion of factors, as discussed above, which together exhibit pro-tumorigenic effects³⁸⁶. MSCs already located in the bone may therefore be activated by the bone metastasis, which subsequently exert the pro-tumorigenic effects. Comparison of MSCs isolated from normal bone and bone metastasis using RNA-sequencing will allow the generation of an 'activated gene signature' in the bone metastatic MSCs, that in turn exhibit pro-tumorigenic effects.

4.3.3 MSC to CAF differentiation

Within the TME, MSCs also undergo fibrogenic differentiation giving rise to tumour-promoting CAFs. We provide preliminary data to demonstrate that MetBo2 cells induce MSC to fibroblast differentiation as MSCs in direct contact with MetBo2 cells display a fibrogenic phenotype, including expression of α SMA. Similar results have also been observed where α SMA expression is induced by tumour-derived molecules^{128,368,387}. This phenotype was dependent on direct contact with the tumour cells as MetBo2-derived conditioned media did not induce α SMA expression in MSCs. We also demonstrate that cells expressing α SMA⁺, FSP⁺ or FAP⁺, defined here as CAFs, are present in breast cancer bone metastasis. α SMA⁺ CAFs within breast cancer bone metastasis may originate from MSCs recruited to the tumour region and is regulated by unidentified direct tumour cell-MSC interactions.

α SMA, considered a specific marker for myofibroblasts, is one of the most widely used markers for CAFs and is upregulated in CAFs compared to normal fibroblasts^{282,388–394}. FSP and fibroblast-activated protein FAP have also been used to identify CAFs^{125,127,129,395–398}, although FAP has also found to be expressed in a limited number of tumour cells^{399,400}. Other non-specific markers expressed by CAFs include PDGFR α and ECM proteins such vimentin, tenascin C, desmin and perostin³⁹³. Whilst no specific marker for CAFs is known, a combination of these additional markers described may identify the CAF population in breast cancer bone metastasis. Due to the controversy and overlap between the identities of mesenchymal cells, it is also crucial to investigate whether these cells exclusively express the markers as a distinct population, or whether there is overlap in the expression of the markers. Further immunostaining and flow cytometry of these fibroblast markers as well as MSC markers described previously should therefore be performed. The mesenchymal compartment of the TME can be identified in flow cytometry using CD45/CD11b⁻ CD31⁻ Ter119⁻ to eliminate immune cells, endothelial cells and erythroid cells, respectively. The use of fluorescently labelled tumour cells means they also can be gated against. The remaining population of cells can then be further analysed using mesenchymal markers such as PDGFR β , NG2, CD146, Nestin-GFP, FSP and FAP. This will further characterise the populations of mesenchymal cells within breast cancer bone metastasis.

Furthermore, we demonstrate that fibroblasts also promote the growth of MetBo2 cells. NFs and CAFs were previously isolated from patients undergoing breast reduction or surgery for breast cancer removal, respectively and were immortalised to create a cell line¹⁶⁸. Characterisation using immunostaining demonstrates that these cells exhibit α SMA⁺ expression^{168,401}. Similar results were observed in both direct co-culture of fibroblasts with MetBo2 and in transwell assays using fibroblast-derived conditioned media. This suggests that soluble factors secreted from the fibroblast may directly act on the tumour cells to promote tumour growth. Both normal fibroblasts and CAFs were able to induce tumour growth, suggestive of a process independent of a 'cancer activated' phenotype, although the tumours cells may activate the normal fibroblasts during the co-culture assay. There are also differences between the NFs isolated from different patients and it is also unclear why NFs from patient 1 were only able to induce growth at a 1:1 ratio. It should also be noted that these fibroblasts were isolated from normal and malignant breast tissue rather than the bone therefore the different origins of fibroblasts should also be considered. Isolation of fibroblasts from metastatic murine bones using a protocol previously used to isolate CAFs from tumour tissue was tested but was unsuccessful due to problems with tumour cell contamination that could not be eliminated⁴⁰².

Nevertheless, together, with the data from the MSC co-culture, we propose a potential model where the direct contact between MSCs and tumour cells induces fibrogenic differentiation of the MSCs to a CAF-like phenotype. In turn, these CAFs secrete soluble factors that act on the tumour cells to promote proliferation and growth. This would explain the requirement for direct co-culture between MSCs and tumour cells to induce tumour cell growth. Cytokines arrays and molecular inhibition should be performed to identify and confirm the function of the molecules responsible for these effects. It is also essential to investigate whether this process occurs within the bone metastasis *in vivo*. Lineage tracing experiments can be performed to determine if MSCs undergo fibrogenic differentiation to give rise to CAFs within the bone metastasis. This involves the injection of fluorescently labelled MSCs into tumour bearing mice and subsequent immunofluorescent co-staining to determine if the fluorescently labelled MSCs also express fibroblast markers, including α SMA, FSP and FAP.

This model is supported by several studies that have demonstrated that MSCs recruited to the tumour microenvironment display fibrogenic differentiation towards

CAFs. Mishra and colleagues¹²⁸ first demonstrated the cancer-induced MSC differentiation to CAFs in breast cancer. Using *in vitro* differentiation assays, they showed that exposure of human bone marrow-derived MSCs to conditioned media collected from breast cancer cells, glioblastoma cells and pancreatic cancer cells induced the expression of α SMA, FSP and vimentin as well as other CAF-associated genes. However, the soluble factors involved in the differentiation were not identified. These tumour-educated MSC, or CAFs, exhibited an increased ability to promote tumour cell growth in the breast cancer model both *in vitro* and *in vivo* by secreting CXCL12 compared to naïve control MSCs. These effects were also specific to cancer-induced fibroblasts as fibroblasts generated by 5-azacytidine-induced differentiation of MSCs exhibited lesser effects and did not secrete CXCL12¹²⁸. Additional studies demonstrate that TGF β 1/SMAD signalling is a major contributor to breast cancer-induced differentiation of MSC to fibroblast³⁸⁷. TGF β 1 treatment of human bone marrow MSCs induces expression of CAF-markers *in vitro* including FSP1, FAP and α SMA, and inhibition of TGF β 1 prevents cancer cell-induced differentiation *in vivo*⁴⁰³. Breast cancer-derived exosomes induced a CAF-like phenotype in MSCs, including an increased in α SMA expression, via the SMAD-mediated signalling pathway⁴⁰⁴. Accumulating evidence suggests that breast cancer-derived osteopontin also drives integrin-dependent TGF β 1 signalling in MSCs, which mediates differentiation to CAFs. MDA-MB-231 breast cancer cells, which highly express osteopontin, induce this process, whereas MCF7 cells expressing low levels of osteopontin cells do not. Inhibition of integrins, α v β 3 and CD44, on the surface of the MSCs attenuated the response to TGF β 1 and prevented the differentiation. Additionally, MSCs isolated from the liver metastatic site express CAF markers, including α SMA and CXCL12, which was regulated by osteopontin. *In vivo* studies using tumour xenografts demonstrate that osteopontin increases tumour growth and metastasis by regulating the MSC to CAF differentiation in a TGF β 1-dependent manner⁴⁰⁵.

Similar MSC-to-fibroblast differentiation has been observed in many other cancer models including pancreatic, ovarian, gastric and prostate cancer^{129,329,368,406}. Quante *et al.*³⁶⁸ determined that at least 20% of CAFs within the TME originated from bone marrow-derived MSCs in an *in vivo* murine model of inflammation-induced gastric cancer. These CAFs subsequently expressed IL6, Wnt5a and BMP4, and promoted tumour growth³⁶⁸. Similarly, MSC to CAF differentiation has also been observed in a prostate cancer model. MSCs are recruited to the primary tumour and differentiate to CAFs in a CXCL16-CXCR6-dependent manner. qRT-PCR and immunostaining reveals

that FSP, α SMA and vimentin are all increased in the MSCs treated with prostate cancer cell-derived conditioned media, and is attenuated in MSCs isolated from CXCR6^{-/-}-deficient mice. In addition, reduced α SMA⁺, FSP1⁺ and vimentin⁺ cells were observed in prostate cancer tumours established in CXCR6^{-/-} compared to wildtype littermate controls. These MSC-derived CAFs subsequently secrete CXCL12 that interacts with the tumour cells via CXCR4 and induces the epithelial-to-mesenchymal transition (EMT) and metastasis to the bone¹²⁹. It should be noted that whilst breast and ovarian cancer cell-derived factors induce CAF differentiation of bone marrow-derived MSCs, this effect is not observed in human umbilical cord Wharton's jelly MSCs. This highlights the specific effects between the tissue origin of MSCs and suggest the effect of cancer on fibroblast differentiation is unique to bone marrow-derived MSCs⁴⁰⁷.

A recent study also demonstrated that PDGFR β ⁺ NG2⁺ pericytes also provide a source of CAFs in a model of squamous carcinoma, termed as a pericyte-to-fibroblast transition. Targeting of the PDGF-BB-PDGFR signalling ablates the abundance of intratumoural FSP⁺ and α SMA⁺ fibroblasts. This effect was confirmed using shRNA-mediated genetic knockdown and pharmacological inhibition of PDGFR β , and by establishing tumours in PDGFR β -depleted mice. The pericyte-to-fibroblast transition was mediated by PDGF-BB, which induced FSP1 expression and an elongated, spindle-like shape similar to CAFs, whilst downregulating NG2 expression. This effect was abrogated by inhibition PDGFR β , but not PDGFR α , indicating the role of the former in the signalling. Lineage tracing experiments were also performed by establishing tumours in TN-AP-CreERT2^{-/-} and NG2-CreERT2^{-/-}:R26R-tdTomato mice in which ALP⁺ and NG2⁺ pericytes are labelled, respectively. Immunostaining demonstrates that the tdTomato-labelled pericytes were associated with tumour vasculatures, whereas PDGF-BB treatment induces a loss of AP and NG2-expressing pericytes. Remaining pericytes exhibited elongated morphologies distinct from the blood vessels, and gained expression of FSP1 and PDGFR α . This confirms NG2⁺ expressing pericytes differentiate into fibroblast in a PDGF-BB-dependent manner. To complement the murine studies, pericytes isolated from fresh human samples based on their NG2 expression also undergo PDGF-BB-induced differentiation to a α SMA⁺, FSP1⁺ CAF-like phenotype. Genome-wide profiling of human tumours also determines a CAF-like gene signature similar to those observed *in vitro*. Finally, these pericyte-sourced CAFs promote tumour growth, angiogenesis, invasion and metastasis. In addition, the expression of FSP1 is increased in PDGFB-high cancers compared to

PDGFB-low cancers, and both FSP1 and PDGFB expression is negatively correlated with survival in colorectal cancer, glioblastoma and lung cancer patients¹²².

Whilst a similar process of MSC to CAF differentiation is observed in breast cancer bone metastasis, it is important to highlight that previous studies identify soluble factors responsible for MSC to CAF differentiation. In contrast preliminary data demonstrates that direct contact between MSCs and MetBo2 cells is required for CAF differentiation. This may imply a mechanism specific to breast cancer bone metastasis; however, this requires further investigation and confirmation *in vivo*.

4.3.4 MSC-macrophage interactions in bone metastatic growth

MSCs also modulate components of both the innate and acquired arm of the immune system to create an overall immunosuppressive environment, which permits evasion of the immune system and promotes tumour growth. We therefore investigated the immunosuppressive potential of bone-marrow derived MSCs with a particular focus on tumour-associated macrophages. Another project in the lab demonstrates that a distinct population of CD204⁺ IL4Rα⁺ macrophages, known as BoMAMs, exist within the same breast cancer bone metastasis model. BoMAMs display upregulation of IL4Rα, CD204, PD1, Tim4 and PDL1, and downregulation of MHCII to create an immunosuppressive phenotype. They are recruited to the tumour region in a CCR2-dependent manner, where they exhibit a specific gene signature and pro-tumorigenic effects. It is demonstrated via several macrophage depletion models that ablation of these macrophages reduces bone metastasis growth (Ma *et al.* submitted). We hypothesised that MSCs may also exert their growth-promoting effects by inducing recruitment and polarisation of macrophages to generate these BoMAMs.

Several pilot experiments were performed to investigate the effect of MSCs on macrophages within the bone and provide preliminary *in vitro* data that bone marrow MSCs alter the phenotype of bone marrow macrophages. We first show that macrophages cultured both in MSC-derived conditioned media and in direct co-culture with MSCs at a 1:1 ratio results in a morphological change. Macrophages cultured alone predominantly exhibit a rounded phenotype, whilst the MSCs induce filopodia and increased spreading of the macrophages. Whilst the morphology of macrophages cultured with MSCs has not been previously reported, Patel *et al.* demonstrated that macrophage morphology and function was modified by the adhesion to a substrate. This results in actin polymerisation and increased rhoGTPase activity, thus

adhesion^{288,408}. We therefore hypothesise that secretion of ECM proteins from the MSCs, including fibronectin and tenascin C as previously reported⁴⁰⁹ may promote adhesion and spreading of the macrophages. We further demonstrate that MSCs enhance the migration of macrophages via secretion of unidentified chemoattractants. Together, with the increased filopodia observed *in vitro* in the culture with MSCs, this may promote migration, invasion and recruitment of macrophages to the tumour microenvironment. Several other studies also demonstrate that MSCs can promote recruitment and activation of myeloid cells^{316,326}. For example, TNF α -activated MSCs also recruit monocytes and macrophages to lymphoma in a CCL2-CCR2-dependent manner³⁸⁵.

We next demonstrated that MSCs induce a TAM-like phenotype in the macrophages similar to that exhibited by the tumour-promoting BoMAMs in the same model (Ma *et al.*, submitted). This includes the upregulation of IL4R α , CD204 and PD1 via the direct co-culture with MSCs. Furthermore, MSC-derived conditioned media had no effect on PD1 and IL4R expression, whilst ablating CD204 expression. This suggests that the direct contact between MSCs and macrophages induces the upregulation of these surface molecules. Previous studies demonstrate that macrophage polarisation is regulated by integrin-mediated interactions between cells⁴¹⁰, thus may play a role in MSC-induced polarisation. In contrast, MSCs promote a reduction in PDL1, Tim4 and MHCII expression. Soluble factors contribute more to this interaction as MSC-derived conditioned media have a more significant effect than direct culture with MSCs. It is unclear why conditioned media had a greater effect than direct co-culture as MSCs within the culture would also secrete similar soluble factors. It is plausible that the macrophages may inhibit secretion of MSC-derived soluble factors, but would need further investigation. Whilst PDL1 and Tim4 expression is upregulated in BoMAMs isolated from breast cancer bone metastasis, we observed a decrease of these molecules in MSC-treated macrophages. The *in vitro* assay did not consider the tumour aspect of the TME, and therefore cancer-associated MSCs or macrophages may display different effects. Conversely, MSCs may not contribute to the upregulation of PDL1 and Tim4 in BoMAMs with another cell type being responsible. MSCs may also downregulate PDL1 and Tim4 in an alternative macrophage population within the breast cancer bone metastasis. Nevertheless, the overall change of phenotype in MSC-educated macrophages suggests that MSCs induce a pro-tumorigenic, immunosuppressive macrophage population.

To further investigate the role of MSCs in inducing a growth-promoting BoMAM-like phenotype, further experiments are required to confirm the effects of MSCs on macrophages *in vivo*. Immunostaining and flow cytometry analysis of these macrophage-associated markers will be performed in metastatic bones collected from in nestin⁺ MSC-depleted mice to determine the effect of nestin depletion on macrophage abundance and phenotype. We hypothesise that MSC depletion will result in a reduction of these TAM-like macrophages in the breast cancer bone metastasis. The involvement of macrophages in MSC-induced metastatic growth could also be confirmed by injection of MSCs into tumour bearing mice with and without macrophage depletion. It is expected that MSCs would promote tumour growth in control mice, whilst these effects would be attenuated in macrophage depleted mice. To support the *in vivo* findings, *in vitro* cultures would also determine the effects of MSC-educated macrophages, which have been exposed to MSCs or MSC-derived conditional media, on tumour cell growth, in comparison to the effects of naïve, uneducated macrophages. We hypothesise the MSC-educated macrophages would enhance tumour cell growth to a greater extent than the uneducated macrophages, which would determine the contribution of MSC-educated macrophages in bone metastatic growth. Comparison of the gene expression between the MSC-educated macrophages and naïve, uneducated macrophages using qPCR may also offer insight into the molecular changes involved in the tumour-promoting effects. The *in vitro* studies could also be repeated using cancer-associated MSCs isolated from metastatic bones to determine if a similar effect occurs in the TME. We will further characterise the molecular interactions between MSCs and macrophages using RNA-sequencing and qPCR of MSCs and macrophages. Potential mechanisms will be tested functionally by performing a similar macrophage-differentiation assay in the presence of molecular inhibition or knockdown. Lastly, this process should be confirmed in a human model of breast cancer bone metastasis by repeating similar *in vitro* and *in vivo* experiments using human-derived MSCs and macrophages. In addition, bioinformatic analysis of datasets from human samples may provide evidence of similar effects occurring in the development of cancer in patients. This may include correlation between MSC abundance and macrophage phenotype. Overall, it is clear that MSCs promote a phenotype similar to M2-like immunosuppressive, tumour-promoting BoMAMs¹³³. This includes the upregulation of PD1, CD204 and IL4R α , and downregulation of MHCII.

There is now an increasing amount of evidence to suggest that TAMs and mesenchymal cells exhibit pro-tumorigenic effects in a synergistic, cooperative manner.

Firstly, TAMs and MSC/CAFs are often located in the same region of the TME and the abundance of each cell type is positively correlated with each the other in several cancers, including breast cancer, oral squamous cell carcinoma, colorectal cancer and neuroblastoma^{119,282,411–415}. We similarly observe a comparable location of macrophages and MSCs in immunostaining staining in breast cancer bone metastasis (data not shown). However, this staining was not performed on the same section and therefore needs to be confirmed using co-staining. Furthermore, whilst we also demonstrate that CD146 abundance is not correlated with Iba1 abundance, a similar correlation should be calculated with alternative markers of MSCs, such as nestin⁺ MSCs, which have been previously shown to interact with macrophages in the bone⁸². The presence of mesenchymal cells and TAMs are also synergistically associated with cancer progression and poor prognosis^{282,412–414}. In a murine model of lymphoma, co-injection of lymphoma-derived MSCs with cancer cells promoted tumour growth and was associated with increased recruitment of CD11b+Ly6C⁺ monocytes, F4/80⁺ macrophages and CD11b⁺ Ly6G⁺ neutrophils to the tumour. The pro-tumorigenic effects of the MSCs could be abrogated by the depletion of macrophages as well as CCR2^{-/-} mice, demonstrating that cancer-associated MSCs promote tumour progression by the recruitment of macrophages. These effects were not observed using normal BM-MSCs, but could be activated to have a similar cancer-associated phenotype with TNF α treatment. This suggests TNF α may be released from the tumour cells to activate the MSCs, but is not tested functionally. Together with the correlation between MSCs/CAFs and macrophages observed in human tissues, this suggests that role and the molecular mechanisms of the MSC/CAF-macrophage interaction is important in tumour growth and requires further investigation³⁸⁵.

The effect of MSCs on macrophages is well studied in normal tissues, and overall induce an immunosuppressive phenotype⁴¹⁶. One of the first reports of MSC-macrophage interaction was identified in macrophages isolated from human peripheral blood and co-cultured with human bone marrow-derived MSCs. MSC-educated macrophages displayed an M2 phenotype and an increased phagocytic activity⁴¹⁷. MSC-derived factors, including TGF β , and HGF, were shown to modulate macrophages as well as other components of the immune system^{418,419}. Subsequently, MSC-educated macrophages have been shown to produce several cytokines including TNF α , IL10 and CCL8, which are involved in tumour development⁴²⁰.

Following this, increasing evidence demonstrates that both MSCs and CAFs recruit macrophages and induce a similar immunosuppressive effect in several cancer types. Wolfe *et al.*¹¹⁸ previously demonstrated in a model of SUM146- and MDA-IBC3-derived breast cancer that MSCs induced an M2-phenotype in an IL6-dependent manner. Exposure of macrophages to MSC conditioned media resulted in enhanced CD206 and arginase-I expression *in vitro* suggestive of an M2-phenotype, and that these MSC-educated macrophages promoted breast cancer cell migration and self-renewal compared to naïve macrophage controls. These effects were dependent on MSC-derived IL6 and were inhibited by IL6-neutralising antibody. This *in vitro* work was supported by *in vivo* models, which demonstrated that anti-CSF1 treatment abrogated the tumour-promoting effects of MSCs, including reduced breast cancer growth and invasion after co-injection of breast cancer cells and MSCs. The M2 macrophages subsequently promoted migration of MSCs, and were required for MSC-regulated invasion of BCa¹¹⁸. This work is further supported by Jia *et al.*⁴²¹ who demonstrated in an alternative model of MDA-MD-231-derived breast cancer that MSC-derived IL6 polarised TAMs into M2-like phenotypes, whilst CCL2 from MSCs induced macrophage recruitment⁴²¹. The role of IL6 as well as IL8 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) is also important in MSC-induced M2 polarisation of macrophages in gastric cancer^{422,423} and colorectal cancer^{424,425} where IL6/IL8-stimulated JAK2/STAT3 signalling resulting in the polarisation. Subsequently, these macrophages promoted metastasis by enhancing EMT and migration⁴²². In a model of colorectal cancer, CAF-derived IL8 induced monocyte recruitment to the tumour, which are subsequently polarized into an M2 macrophage phenotype by CAF-derived IL6. Importantly, these TAMs and CAFs both suppress NK cell function further creating an immunosuppressive microenvironment. CAFs also promote M2 macrophage recruitment *in vivo* as co-injection of bone marrow-derived MSCs with tumour cells results in increased tumour growth with increased macrophage infiltration, whilst macrophage depletion ablates the pro-tumorigenic effects of the MSCs⁴²⁴.

CCL2 and CXCL12 have also been implicated in CAF-induced monocyte recruitment and M2 polarisation in breast cancer. CAFs isolated from breast cancer patients, which exhibit increased α SMA compared to normal fibroblasts, induced an M2 phenotype in macrophages. This included PD1 expression, increased anti-inflammatory IL10 and decreased pro-inflammatory IL12, which together creates an immunosuppressive environment. Subsequently, these CAF-educated macrophages suppressed T cell proliferation as well as directly promoting tumour cell proliferation and migration and via

EMT induction. Importantly, these effects were not observed with normal fibroblasts suggestive of a tumour-dependent factor²⁸². Conversely, CXCL12-CXCR4, but not CCL2 or IL6, is important in CAF-induced monocyte migration and macrophage polarisation in prostate cancer. This highlights the intertumoural heterogeneity and importance of investigating each tumour type and metastasis location separately⁴²⁶. These MSC-educated macrophages promoted tumour cell migration as well as endothelial tube formation suggestive of pro-angiogenic effects. Furthermore, CAF-educated M2 macrophages activate normal fibroblasts into CAF-like, demonstrating the complexity and bidirectional signalling in the TME⁴²⁶. Mathew *et al.*⁴²⁷ demonstrated that MSCs also enhance pancreatic cancer growth by inducing infiltration and polarization of macrophages into an alternatively activated, tumour-promoting phenotype. This effect was restricted to MSCs isolated from pancreatic carcinoma, which expressed IL6 and IL10 that could be inhibited to prevent macrophage polarization⁴²⁷.

Another molecule implicated in CAF-induced macrophage recruitment and M2 polarisation is Chitinase 3-like1 (Chi3L1). CAFs isolated from the PyMT breast cancer model exhibited increased Chi3L1 expression compared to normal fibroblasts. This expression was induced in normal fibroblasts by tumour cells demonstrating a role of the cancer cells in the paracrine loops. *In vitro* assays demonstrated that CAF-derived Chi3L1 polarised Raw264.7 macrophages to an M2-phenotype and activated the MAPK and Pi3K pathways. Injection of tumour cells with CAFs or recombinant Chi3L1 resulted in increased TAM infiltration, whilst injection with Chi3L1-knockout CAFs reversed this recruitment and M2 polarisation. In addition, CAF-derived Chi3L1 inhibits T cell infiltration and promotes a type 2 inflammatory status in the TME, which was again reversed after co-injection with Chi3L1 knockdown CAFs. Furthermore, CAFs have direct pro-tumoral effects including promotion of tumour cell migration in both scratch assays and transwells assays (but not proliferation) in a Chi3L1-dependent manner and was abrogated in Chi3L1 knockdown fibroblasts. In summary, tumour-derived factors induce secretion of Chi3L1 from CAFs, which in turn, supports tumour progression directly by enhancing tumour cell migration as well as the promotion of the immunosuppressive tumour microenvironment. This includes the recruitment and M2 polarisation of macrophages, inhibition of T cell recruitment, and an induction of a type 2 inflammation in the TME⁴²⁸.

The release of exosomes containing TGFβ1, C1q and semaphorins from MSCs also promotes an M2-like macrophage phenotype in a breast cancer model. Intratumoural injection of MSC-derived exosomes into established tumours resulted in an increased abundance of CD206⁺ macrophages, reduced CD4⁺ and CD8⁺ T cell-derived IFNγ, and increased PDL1 and PD1 in the macrophages and tumour cells, respectively. This immunosuppressive phenotype not only increased tumour growth compared to PBS-only controls, but tumours also exhibited increased EMT. Furthermore, the depletion of macrophages reversed the immunosuppressive and pro-tumorigenic effects of MSCs. This is key study demonstrating the involvement of MSCs in the regulation of the PDL1-PD1 checkpoint pathway¹¹⁹.

In a model of pancreatic carcinoma, CAFs also induce M2 macrophage polarisation. CAF-derived soluble factors induced M2 polarisation in monocytes, which was abolished by using ROS-depleted CAFs and using neutralising anti-M-CSF antibody. Further studies demonstrate that the secretion of M-CSF from CAFs induced ROS production in the macrophages leading to the polarised phenotype⁴²⁹. In a murine liver cancer model, it was demonstrated that specifically αSMA⁺ FAP⁺ CAFs, which were activated by STAT3 signalling, created an immunosuppressive environment by inducing the recruitment of myeloid cells, resulting in promotion of tumour development. This was in a CCL2-CCR2 manner where CAF-derived CCL2 promoted recruitment of CCR2 monocytes, and was abrogated in CCR2^{-/-} deficient mice, resulting in reduced tumour volume⁴³⁰.

A complex integration of paracrine loops between breast cancer cells, MSCs and TAMs was recently identified. MSCs are recruited to breast cancer tissue by CXCL16-CXCR6-mediated interactions with the tumour cells. These MSCs in turn secrete CCL5 that binds to CCR5 on breast cancer cells. This induces that secretion of cancer cell-derived CSF1, which drives the recruitment of TAMs. Whilst both signalling interactions function in an independent manner, both are dependent on the transcriptional activity of HIF, which is activated by the hypoxic environment⁴³¹. This demonstrates the ability of MSCs to regulate TAMs in an indirect manner by altering other components of the TME.

The interaction between macrophages and MSCs has also been shown to be important in models of inflammation outwith cancer models, including model of cardiac tissue repair, lung injury, septicaemia, and colonic inflammation^{432–436}. In cardiac repair, MSC-

derived IL6 also induced a M2-like phenotype including increase anti-inflammatory IL10 and VEGF and decreased pro-inflammatory cytokines including IL1 α , TNF α , IL17 and IFN γ ⁴³². Similar results were observed in lung injury where MSC-derived conditioned media inhibited the production of TNF α from macrophages to protect the lung tissue from further injury⁴³³. MSCs also induce IL10 from macrophages to protect from septicaemia-induced damage, and could be reversed by IL10-IL10R inhibition. Interestingly, this was dependent on activation of TLR4, myeloid differentiation primary response gene 88, TNF1 α and COX2 in the MSCs, suggestive of a role in stimulating the MSCs first. MSCs reprogrammed the macrophages by releasing prostaglandin E₂ (PGE₂), which acts on macrophages through the prostaglandin receptors, EP2 and EP4⁴³⁴. Liu *et al.*⁴³⁵ further demonstrated in a model of colonic inflammation that MSC-derived exosomes containing metallothionein-2 induce a M2 phenotype in macrophages, which subsequently release IL10 that inhibits CD4⁺ T cell proliferation and release of T-cell derived cytokines. MSC-derived exosomes also reduce inflammation *in vivo* and could be blocked by macrophage depletion⁴³⁵. MSC-derived CCL2 was also required to induce macrophage recruitment in a wound healing model. MSCs isolated from CCL2-deficiency mice were unable to recruit macrophages resulting in reduced wound healing capability⁴³⁷. MSC-educated macrophages here also exhibit increased IL10 and IL6 and decreased IL12 and TNF α in response to lipopolysaccharide stimulation²²⁷. Other studies demonstrate that bone marrow-derived MSCs exhibit a downregulation of TNF α , IL6 and IFN γ , and upregulation of IL10 after exposure to lipopolysaccharide-stimulated macrophages⁴³⁸. Similar effects may also occur within the TME and offer insight into potential mechanisms.

Moreover, crosstalk between mesenchymal stromal cells and the other immune cells can create an immunosuppressive environment that permits tumour growth and survival by modulating different components of the immune system. Injection of MSCs into breast cancer-bearing mice also resulted in decreased NK cells and CD8⁺ T cells, increased CD4⁺ Foxp3⁺ cells, increased serum levels of Th2 cytokines, decreased Th1 cytokines and increased CD4⁺ T cell-derived IL10⁴³⁹. In a melanoma subcutaneous model, MSC-mediated reduction of T cells was necessary for tumour growth⁴⁴⁰. The immunosuppressive effects of reduced neutrophils and T cells were also associated with metastasis to the lung^{380,439}. MSCs also modulate other immune cells including B cells, NK cells and dendritic cells to create an immunosuppressive environment, which may also occur in the TME^{441,442}. The interactions between mesenchymal stromal cells

and the immune system are complex and more information can be found at ^{441,442}.

Research of the immune-modulatory effects of mesenchymal cells in the context of bone metastasis is would therefore be valuable.

In summary, MSCs can create an immunosuppressive, tumour-promoting environment, particularly by inducing an M2-like phenotype similar to TAMs in macrophages, as summarised in figure 4.3.2. *In vitro* assays have demonstrated that MSC/CAF-derived conditioned media or co-culture with macrophages induces the M2 phenotype, including PDL1, creating an immunosuppressive phenotype. This was further confirmed using *in vivo* models where co-injection of MSC/CAFs results in increased tumour growth and metastasis, and higher abundance and infiltration of M2-like TAMs. The depletion of macrophages also abolishes the pro-tumorigenic effects of the MSC/CAFs further confirming the mechanism. Due to this immunosuppressive effect of MSCs, it would be interesting to analyse the influence of MSC-educated macrophage

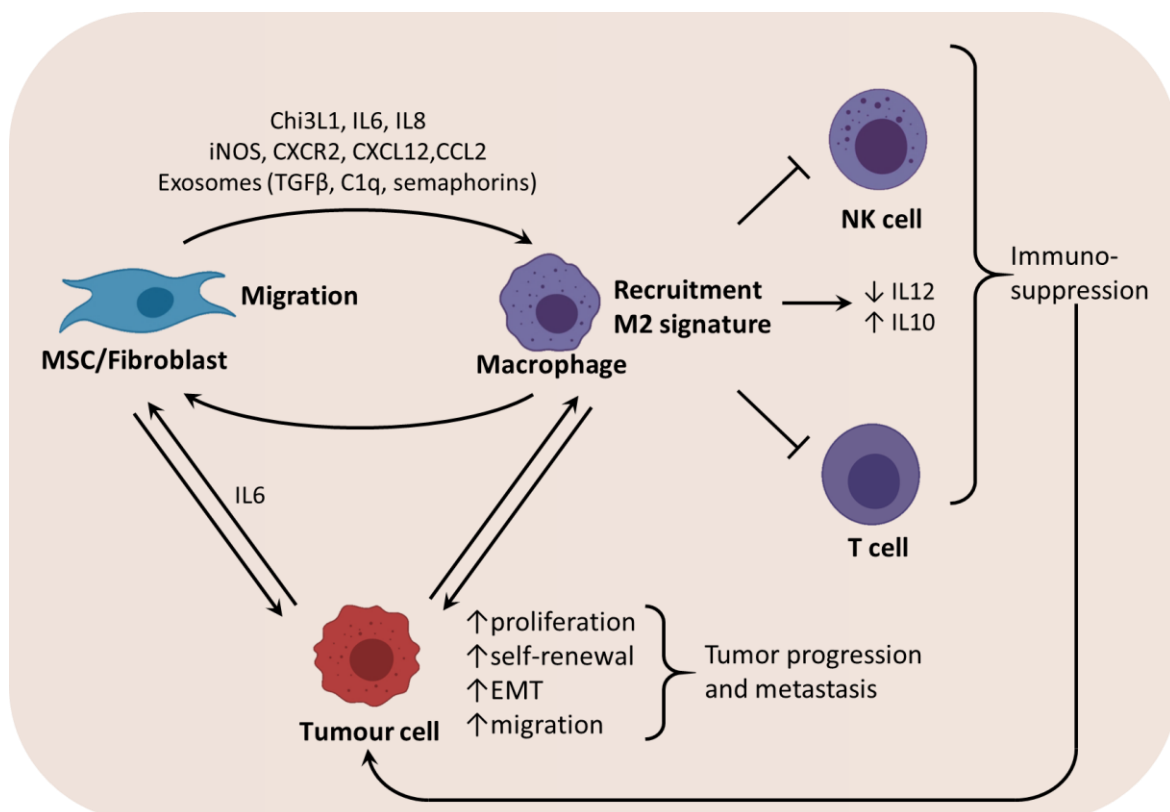


Figure 4.3.2: MSC-macrophage mediated tumour growth and progression. MSCs secrete factors that induce recruitment of macrophages and promote polarisation to a M2-like phenotype. These MSC-educated macrophages inhibit T cells and NK cells to create an immunosuppressive environment, which in turn permits further tumour growth, migration and metastasis.

phenotype on other components of the TME that mediate immunosuppression in the breast cancer bone metastasis model. Furthermore, the expression of each surface protein on TAMs plays independent different roles in promoting tumour growth, as discussed below.

4.3.4.1 PD1-PDL1

PD1, also known as CD279, is an immune checkpoint surface protein involved in the suppression of the immune system by dampening the cytotoxic T cell response^{443,444}. It was initially identified in 1992 by Tasuku Honjo and colleagues⁴⁴⁵ and subsequent studies using PD1-deficient mice defined its role as a negative regulator of T cell mediated immune responses⁴⁴⁶. PD1 has two known ligands, PDL1 (also known as B7-H1 or CD274) and PDL2 (also known as B7-DC or CD273). PD1 and PDL1/2 signalling functions as an important immune checkpoint that negatively regulates the immune response. The interaction between PD1-PDL1 activates downstream signalling of PD1 and suppresses T cell activation, which prevents effector T cell responses and regulates peripheral T cell tolerance. This protects tissues from immune-mediated tissue damage by negatively regulating T cell-mediated immune responses in peripheral tissues and is mediated by the production of cytokines, including IFN γ , TNF α and IL2^{443,447}.

In the last few decades the role of PD1, as well as CTLA4, in the progression of cancer has become increasingly apparent. Cancer cells, as well as components of the TME, exploit PD1 signalling to create an immunosuppressive phenotype and thus is utilised by cancer cells to enhance immune evasion. Tumour cells express ligands for PD1, particularly PDL1, which interacts with PD1 highly expressed on tumour-infiltrating lymphocytes⁴⁴⁸. This results in inhibition of T cell proliferation and cytokine release, and induction of T cell dysfunction, exhaustion and apoptosis^{449,450}. As cytotoxic CD8⁺ T cells are pivotal for killing tumour cells, the activation of PD1 by tumour cells prevents cancer cell elimination. This role of PD1-PDL1 signalling has now been demonstrated in numerous cancer models, including ovarian, lung and renal cancer, and melanoma, and the understanding of the PD1-PDL1 regulated immunosuppression in cancer has substantially increased. This has led to the development of several therapeutics that target the pathway, defined as immune checkpoint inhibitors. Neutralisation of this pathway using specific inhibitors enhances T cell-mediated responses against the tumour cells and by counteracting the inhibitory and suppressive mechanisms of the T cell response, this leads to elimination of cancer. Many of these novel treatments

demonstrate significant clinical efficacy in treating and improving survival rates of many different types of cancer^{443,449}. This includes PDL1 inhibitors, Atezolizumab, Durvalumab, Nivolumab, Pembrolizumab, Cemiplimab and Aleumab, which have shown clinical success and have been FDA-approved for treatment of many cancers including breast cancer, melanoma, renal cell carcinoma, gastric cancer, cervical cancer and non-small cell lung carcinoma (NSCLC), as well as haemological cancers such as lymphoma. The effects on many other cancer types are being demonstrated in ongoing clinical trials⁴⁴³. These treatments contribute to the developing cancer immunotherapy field, which is an emerging method to treat cancer by enhancing or modulating the host immune system. Due to the success of these immune checkpoint inhibitors, the MSC-mediated upregulation of PD1 in the macrophages is of significant interest.

PD1 was initially identified as a co-inhibitory molecule on T cells, predominantly expressed on memory T cells in peripheral tissues. It has also since been found to be expressed on B cells, activated monocytes, macrophages, dendritic cells and natural killer cells^{443,448,451–453}. Whilst the role of the PDL1 and PD-1 interaction in regulation of T-cell suppression in tumour progression is well established, there is limited knowledge about PD1-PDL1 signalling in macrophages and how these signals may affect the function of TAMs. T cells and macrophages are a component of the adaptive and innate immune response, respectively, and display significant differences in function and activation⁴²².

Gordon *et al.*⁴⁵⁴ provided one of the first studies researching PD1-PDL1 in TAMs. They demonstrated that PD1 expression is increased in intratumoural TAMs compared to other tissues such as the spleen and peripheral blood in a murine model of primary colon cancer, and increases with tumour progression and stage in human samples. There is also a clear correlation between high abundance of PD1⁺ macrophages in cancer and poor prognosis. It is particularly higher in M2 macrophages compared to M1 macrophages in human samples, and is also correlated with increased CD206 and decreased MHCII expression, of which the latter was observed in BoMAMs in our model. PD1 expression also negatively correlates with the ability of TAMs to phagocytose tumour cells, and PD1 inhibition *in vivo* increased macrophage phagocytosis, resulting in attenuated tumour growth and increased survival of the mice. This suggests that PD1 prevents macrophage-mediated phagocytosis of tumour cells contributing to immune suppression and evasion⁴⁵⁴. This is supported by similar

results in the metastatic lung of osteosarcoma where there is a high abundance of PD1⁺ TAMs infiltrated in human samples. Anti-PD1 treatment resulted in reduced metastatic lesions in the lung, enhanced tumour cell apoptosis and decreased proliferation. This anti-tumour effect was attenuated by macrophage depletion confirming a role of macrophage-expressed PD1. In addition, whilst PD1 was also expressed on NK cells, the efficacy of anti-PD1 treatment was not compromised by NK depletion, implicating that they were not involved⁴⁵⁵. PD1-expressing macrophages also secrete several cytokines including IL6 in melanoma, particularly after anti-PD1 treatment, resulting in an abrogated CD4⁺ Th1 response, and therefore continued tumour growth⁴⁵⁶. More recently, it was demonstrated that PD1 is upregulated in TAMs compared to normal macrophages in *Kras*-driven pancreatic ductal adenocarcinoma, and engages directly with PDL1⁺ expressing T cells. This interaction results in polarisation of macrophages toward an M2-tolerogenic phenotype via the activation of STAT6 and Akt signalling. This data was confirmed by the increased abundance of activated, anti-tumour macrophages in mice with conditional deletion of PDL1 or PD1 in T cells or macrophages, respectively⁴⁵⁷. Together, these studies demonstrate that macrophage-expressed PD1 promotes tumour growth and progression by preventing immune recognition and phagocytosis of cancer cells, and by inducing T cell suppression at both the primary and metastatic site.

The role of the PD1-PDL1 signalling pathway in bone metastasis has begun to be explored. Zhang *et al.*⁴⁵⁸ demonstrated that bone metastases of renal cell carcinoma highly expressed PDL1 in comparison to other metastatic sites, and was associated with poor overall survival. However, the location of PDL1 expression was not analysed⁴⁵⁸. More recently, PD1 has been implicated in bone metastasis of Lewis lung carcinoma. Both increased PDL1 and PD1 was observed in the tumour microenvironment of bone metastasis. Whilst PDL1 expression was observed on the tumour cells, and confirmed in *in vitro* cell lines, flow cytometry and immunostaining demonstrated that PD1 was increased on both monocytes and macrophages in bone metastasis, compared to contralateral cancer-free bone marrow. In comparison to wild-type controls, *pd1*^{-/-} mice exhibited attenuated bone metastasis progression, including reduced bone destruction, less risk of fractures and decreased cancer-induced pain. Similar results were observed by anti-PD1 antibody, nivolumab, treatment in tumour bearing mice. *In vitro* assays demonstrated that tumour-expressed PDL1 contributed to bone metastatic growth by interacting with macrophage-expressed PD1, which resulted in promotion of RANKL-induced osteoclastogenesis via c-Jun N-terminal kinase (JNK)

activation *in vivo*. This subsequently enhanced bone metastasis progression by inducing further osteolysis. PDL1 may also increase CCL2 expression from the macrophages, which acts in an autocrine manner to activate CCR2 receptors to mediate macrophage to osteoclast differentiation⁴⁵⁹. Similar effects may also occur in breast cancer bone metastasis, where an MSC-induced increase in PD1 expression on macrophages results in differentiation to osteoclasts, and subsequently contributes to the osteolytic growth cycle.

Data from within the lab demonstrates that PD1/PDL1 inhibition also significantly reduces breast cancer bone metastasis, although the target cells have not yet been investigated (unpublished). We demonstrate that PD1 is expressed in BoMAMs in metastatic breast cancer (Ma *et al*, submitted), and that MSCs induce PD1 expression in macrophages. This is supported by Yavuz *et al.*²⁸² who previously demonstrated that PD1 expression on TAMs is regulated by the mesenchymal lineage in a human model of breast cancer. Monocytes isolated from healthy donors were cultured in conditioned media derived from CAFs isolated from patient breast cancer samples, which leads to an upregulation of PD1, whereas culture with normal fibroblasts did not, suggestive of a tumour-specific effect. This demonstrates that CAFs can induce a pro-tumoral TAM-like phenotype, which exhibit strong immunosuppression, including the induction of PD1 expression²⁸². The mechanisms involved in the regulation of PD1 expression on macrophages remain to be fully elucidated. Whilst our data suggests that direct contact between MSCs and macrophages is required to induce the MSC-mediated increase in PD1, other studies show PD1 expression is increased by soluble IFN γ ⁴⁶⁰ and activation of Toll-like receptor 4 (TLR4) and TLR2 via the Nuclear factor kappa B (NF-KB) signalling⁴⁶¹.

Although the role of PDL1 on macrophages is less studied, there is now an increasing number of studies reporting PDL1 expression in TAMs within several cancer types^{462–464}. As expected, the expression of PDL1 on macrophages is increased with progression in hepatocellular carcinoma and is associated with high mortality⁴⁶⁵. Comparably, it was demonstrated in NSCLC samples that lower numbers of PDL1⁺ macrophages was correlated with better patient survival. Patients with PDL1 expression in <6.3% of TAMs displayed increased overall survival than patients exhibiting expression in >6.3% of TAMs⁴⁶². The abundance of PDL1-expressing TAMs is also predictive of a positive response to anti-PDL1 and anti-PD1 targeted therapies⁴⁶⁶. In a study across multiple cancer types, expression of PDL1 in at least

10% of TAMs resulted in treatment response rates of up to 80%⁴⁶⁷. Other studies demonstrated that despite no or low expression of PDL1 on tumour cells, cancers with abundant levels of PDL1-expressing TAMs responded to PDL1 inhibition. This suggests that PDL1-expressing TAMs contribute to tumour growth and are a key component of the response to anti-PDL1 therapies^{466,468,469}. Anti-PDL1 antibodies targeting TAMs also have therapeutic benefit in PyMT breast cancer and B6 melanoma in a T cell-independent manner⁴⁶⁶. Together, these studies show that inhibiting PDL1 on TAMs inhibits tumour progression, and thus it plays a role in promoting tumour development.

It is expected that PDL1-expressing TAMs function similarly to suppress T cell response to tumours by interacting with PD1 expressed by T cells. This has been exemplified in several studies where TAMs suppress the cytotoxic activity of CD8⁺ T cells through the expression of PDL1. In a model of hepatocellular carcinoma, macrophages within the peritumoural stroma expressed surface PDL1, but not those located amongst the cancer cells. The PDL1 expression was regulated by monocyte-derived TNF α and IL10. The PDL1⁺ macrophages suppressed T cell-mediated responses and promoted tumour growth, which could be prevented by inhibiting PDL1^{465,470}.

Other studies have defined alternative, unique effects of PDL1 expression in macrophages. In a model of lung cancer, it was demonstrated that PDL1 expression of TAMs specifically protected these TAMs from T cell-mediated killing, but did not inhibit the overall T cell response. This protects and maintains tumour-promoting TAMs in the TME. In contrast, PDL1-expressing tumour cells restricted the T cell response to create immunosuppressive environment⁴⁵². Another study investigating the role of PDL1 in regulating the biological functions of macrophages showed that anti-PDL1 treatment of macrophages *in vitro* resulted in increased macrophage proliferation, survival and hypertrophy. They also exhibited increased IL12 and TNF α expression as well as activation of the mTOR pathway. Furthermore, macrophage isolated from PDL1^{-/-} mice exhibited increased proliferation and upregulation of factors including TNF α , inducible nitric oxide synthase (iNOS), IL1 β , CCL7, CXCL3 and CCL2, consistent with an inflammatory phenotype. Using *in vivo* studies of B16 melanoma and PyMT breast cancer, they also demonstrate that anti-PDL1 antibodies result in intratumoural macrophage accumulation, which was independent on T cells as demonstrated in Rag^{-/-} mice. This macrophage accumulation results in increased macrophage-mediated anti-

tumour activity and therefore reduces tumour growth. This data defines a functional role of PDL1 as a negative signalling molecule in macrophages that reduces macrophage-mediated response against the tumour cells and thus permits tumour progression⁴⁶⁶.

However, the role of PDL1 on TAMs is controversial with other studies demonstrating that high PDL1 expression on TAMs and is associated with better prognosis in several cancers. Pollari *et al.*⁴⁶³ demonstrated that the abundance of PDL1⁺ macrophages identified in testicular lymphoma samples correlated with increased PD1⁺ T cells. Both were associated with favourable outcomes and increased survival⁴⁶³. Similar results were observed in hepatocellular carcinoma with the number of PDL1⁺ macrophages being associated with increased survival compared to samples from patients that exhibited PDL1⁻ macrophages⁴⁶⁴. As PDL1 expression on macrophages is correlated with better outcomes, it suggests that PDL1 may play a role in preventing or reducing cancer progression. This would mean that the decrease in PDL1 expression observed after treatment with MSC-derived conditioned media may help bone metastatic growth. Due to the conflicting reports regarding the relationship between PDL1⁺ TAMs and cancer prognosis, and potentially tumour type-specific roles of PDL1 on TAMs, further investigation into the role in bone metastasis is required.

Moreover, whilst PDL1 expression can be constitutively expressed independent of TME due to constitutive expression of oncogenic signalling in some cancer cells such as in Hodgkin lymphoma and gliomas^{471,472}, PDL1 expression is predominantly regulated by exogenous stimuli such extracellular signalling. The expression of PDL1 on macrophages has been shown to be regulated by several cytokines and factors, including TNF α , IFN γ , IL10, IL27, IL4, IL6, IL10, IL13, CXCL8, exosomes, autophagosomes, chitin, osteopontin, COX2 and PGE2^{449,452,478–481,465,466,470,473–477}. Often PDL1 expression is induced by stimuli associated with inflammation, such as IFN γ , and is upregulated by lipopolysaccharides⁴⁵³. Furthermore, within a model of sarcoma, inhibition of IFN γ only partially reduced the expression of PDL1, suggestive of a compensatory or combined mechanisms⁴⁸². The regulation of PDL1 in macrophages by MSCs appears to be mediated by secreted factors. MSC-derived factors involved in regulation of PDL1 expression in macrophages have not been identified, nor have the molecules involved in downregulating PDL1. It is also important to note that the regulation of PDL1 expression is context dependent. For example, IL12 upregulates PDL1 expression on endothelial cells and monocyte-derived macrophages, but

downregulates expression in THP1 monocytes⁴⁸³. This highlights the importance of studying each disease or tissue separately, and the requirement to confirm the effect of cancer-associated MSCs and macrophages in PDL1 expression *in vivo*.

4.3.4.2 MHCII

Major histocompatibility complex (MHC) proteins, known as the human leukocyte antigen (HLA) complex in humans, play a major role in stimulation of the immune response, predominantly T cells activation. They are subdivided into MHC I expressed on all nucleated cells, and MHCII, which is predominantly expressed on antigen-presenting cells, including macrophages, dendritic cells and B cells. MHCII-expressing cells act as antigen-presenting cells, which together with the expression of co-stimulatory molecules and adhesion molecules, stimulates the T cell response. Presentation of foreign antigens such as viral or bacterial peptides causes an immune response resulting in its destruction^{484–486}. A similar process occurs within tumours where display of cancer-associated antigens on antigen-presenting cells, including macrophages and dendritic cells, promotes T cell activation and destruction of the tumour cell^{487,488}. However, accumulation of evidence demonstrates that antigen-presenting cells within the TME, including macrophages, exhibit a downregulation of MHCII resulting in a suppression of the T cell response against the cancer cells¹³³. The MSC-mediated downregulation of MHCII on macrophages may therefore mediate a similar effect to permit tumour growth.

The low expression of MHCII has been observed in TAMs in several cancer models including 4T1 human mammary adenocarcinoma, Lewis Lung carcinoma, melanoma and hepatocellular carcinoma^{489–492}. Wang *et al.*⁴⁹² demonstrated that MHCII^{low} TAMs were involved in tumour progression in a model of hepatocellular carcinoma. Whilst a MHCII^{high} population was observed during early development, MHC^{low} TAMs became predominant as the tumour development progressed. These MHC^{low} TAMs were shown to promote tumour growth by secreting immunosuppressive factors, such as IL10 and TGFβ, which inhibit T cell activation. This subset of TAMs also secreted higher levels of VEGF and MMP9, which facilitated increased invasion compared to MHCII^{high} TAMs⁴⁹². In a murine model of breast cancer, MHC^{low} TAMs were the most abundant in the tumour and displayed a distinct molecular profile compared to MHCII^{high} TAMs. MHCII^{low} express increased M2-associated markers such as arginase1, CD163, stabilin-1 and CD206 and decreased M1-associated genes, such as iNOS, COX2, IL1β, IL6 and IL12β, in comparison to MHCII^{high} TAMs. This determines that MHC^{high} and MHC^{low}

TAMs display an M1- or M2-like phenotype, respectively. Interestingly, increased CCL2, CD204 and IL4Rα were also observed on MHCII^{low} TAMs of which the latter is induced in MSC-educated macrophages in this project. Other secreted factors often implicated in tumour progression, such as TNFα, and chemokines involved in monocyte/macrophage recruitment were also increased in MHCII^{low} TAMs, whilst chemoattractants of cytotoxic lymphocyte were decreased. This also supports that MHCII^{high} TAMs may enhance T cell-mediated destruction of tumour cells, but MHCII^{low} does not. Lastly, MHCII^{low} TAMs were enriched in hypoxic regions and demonstrated enhanced proangiogenic activity⁴⁸⁹. Similarly, in a model of melanoma and colon adenocarcinoma, the TAMs with the lowest expression of MHCII were located within the most hypoxic regions of the tumour⁴⁹³. Moreover, using adoptive transfer experiments, it was also demonstrated that MHCII^{low} macrophages were more likely to be recruited to the tumour where they subsequently exacerbates tumour growth. In another lung cancer model MHCII^{low} TAMs, which displayed expression of M2 markers, resided in hypoxic regions. The reduction in tumour hypoxia achieved by establishing tumours in prolyl hydroxylase domain 2 (PHD2)-haplodeficient mice also led to a reduction in angiogenic activity in the MHCII^{low} TAM subset. It did not affect the relative abundance of MHCII^{low} and MHCII^{high} TAM subsets, nor their M2 marker expression^{490,494}. Similarly, MHCII^{low} TAMs were enriched in the hypoxic tumour regions in a breast cancer model⁴⁸⁹. Bone metastasis is also known to be hypoxic and may therefore be implicated in the downregulation of MHCII expression on TAMs^{495,496}. Interestingly, MSCs are recruited to and exhibit an activated state, which exert pro-tumoral effects in hypoxic tumour regions^{497–500}. They may therefore secrete factors in their activated state that promote the downregulation of MHCII to give rise to the enriched population in the hypoxic region.

Together, these studies demonstrate that MHCII^{low} TAMs play a role in shaping the TME to promote tumour progression, including recruitment of monocytes, promotion of angiogenesis through secretion of proangiogenic factors, and inhibition of T cell activation creating an immunosuppressive environment. The downregulation of MHCII is observed in MSC-educated macrophages and is consistent with the BoMAMs isolated from *in vivo* breast cancer bone metastasis. This suggests that MSCs may promote bone metastasis growth by inducing a MHC^{low} phenotype in macrophages, which display immunosuppressive, proangiogenic effects. Similar MSC-mediated regulation of MHCII has been observed in other cancer types as part of the induced M2 phenotype^{385,416}.

4.3.4.3 Tim4

Tim4 belongs to the T-cell immunoglobulin mucin (TIM) family. Whilst other members of the TIM family are predominantly expressed on T cells, Tim4 is expressed on antigen presenting cells such as dendritic cells and macrophages, and functions as a mediator of immune response, similar to MHC. As a receptor for phosphatidylserine, it plays a particularly important role in the removal of apoptotic cells by phagocyte engulfment through the recognition of phosphatidylserine on the surface of the apoptotic cell. This is vital for the maintenance of tolerance and prevents a response against intracellular antigens released from dying cells, which would result in autoimmune disorders^{501–503}. Overexpression of Tim4 on antigen-presenting cells results in a decreased T cell response in a model of infection^{504,505}.

Tim4 is a relatively newly discovered molecule and its role in cancer is less studied. Nevertheless, recent studies have indicated that Tim4 mediates immune tolerance of cancer⁵⁰⁶. Tim4 was shown to be highly ectopically expressed in colorectal cancer tissues, and was associated with metastasis, increased stage and reduced overall survival in patients. This was supported by pre-clinical studies that demonstrated Tim4-overexpression enhanced colorectal cancer growth *in vivo* due to enhanced VEGF-mediated angiogenesis and increased cancer stem cell (CSC)-like properties⁵⁰⁷. Similar results were also observed in NSCLC where increased Tim4 expression was correlated with poor survival and overexpression promoted tumour cell proliferation and growth⁵⁰⁸. Tim4 expression is also observed in histiocytic sarcomas⁵⁰⁹. However, it should be noted that Tim4 was expressed on tumour cells within these studies, and the role in immunosuppression was not investigated.

Baghdadi *et al.*⁵¹⁰ were the first to demonstrate the role of Tim4 in regulation of phagocytosis by cancer-associated myeloid cells. In a model of B16 melanoma, Tim4 is highly expressed on intratumoural myeloid cells, including TAMs and dendritic cells, and its expression on myeloid cells was induced by danger-associated molecular patterns (DAMPs) released from chemotherapy-damaged tumour cells. It functions to repress the immune response that is activated by chemotherapy-induced apoptotic cancer cells. By uptake and removing the apoptotic cells from the microenvironment through autophagy-mediated degradation, antigen presentation of these cells is reduced and therefore the cytotoxic T-cell mediated response is impaired. This prevents destruction of the tumour and therefore permits tumour growth⁵¹⁰. High levels of Tim4 were also found on TAMs isolated from human glioma samples. These TAMs

phagocytosed phosphatidylserine-expressing T cells and induced tumour-specific Tregs, which suppressed CD8⁺ T cell-mediated response against the cancer cells⁵¹¹. This is supported by Li *et al.*⁵¹² who demonstrated that Tim4 mRNA and protein levels were significantly increased in glioma tumour samples compared to adjacent normal tissue⁵¹². More recently, it was demonstrated that a CD163⁺ Tim4⁺ population of resident omental macrophages promoted metastatic spread of ovarian cancer by creating a premetastatic niche for the cancer cells, promoting growth and promoting a CSC-like phenotype in the cancer cells. Depletion of these macrophage prevented metastasis and enhanced survival⁵¹³. Furthermore, blockade of Tim4 augments efficacy of cancer vaccines by inhibiting the suppression of the tumour-specific T cell response in both gastric cancer and melanoma. This enhances the vaccine-induced anti-tumour response resulting in a significant reduction of tumour growth⁵¹⁴.

Tim4 expression on macrophages is promoted by TGFβ, IL6 and TNFα⁵⁰⁸, which are often abundant in TME and may promote overexpression in cancer⁵⁰⁶. In a model of NSCLC, IL6 within the TME was responsible for upregulation of Tim4 in cancer cells in a NF-KB -dependent manner, although the origin of IL6 was not identified. Together, IL6 and Tim4 promoted migration, invasion and EMT of the NSCLC cells resulting in increased metastasis. The expression of both was also synergistically associated with poor prognosis in cancer patients⁵¹⁵. In contrast, Tim4 was negatively regulated by zinc fingers and homeoboxes 2 (zhx2)⁵¹⁶.

Characterisation of the BoMAMs using flow cytometry and RNA-sequencing demonstrates that Tim4 is upregulated in breast cancer bone metastasis. This is in agreement with previously studies in alternative cancer models and may function to mediate immune tolerance and suppress immune response as discussed above. Conversely to the above tumour studies, we observe a decrease in Tim4 on the MSC-educated macrophages. The *in vitro* polarisation assay did not consider the impact of activation by cancer cells therefore cancer-activated MSCs/macrophages may exhibit different effects. Furthermore, Tim4 may not be regulated by MSCs and instead by molecular factors from alternative cells within the TME.

4.3.4.4 IL4Rα

IL4Rα is now recognised as a marker expressed by M2-like macrophages in inflammatory responses and pro-tumorigenic TAMs. IL4 is an important cytokine component of a Th2-type immune response and stimulates 'alternative activation' of

macrophages^{517,518}. Similarly, IL4 is a regulator of the phenotype in TAMs acting as a potential switch of M2 macrophage polarisation^{519,520}. The effect of IL4-activation on macrophage gene signature is described in detail by Wang and Joyce⁵¹⁸ but predominantly upregulate genes involved in immune responses (TNF α), chemotaxis (CCL2, CCL9 and CCL17) and cell adhesion (cadherin1).

The expression of IL4R α on pro-tumorigenic TAMs has been observed in several cancer models, including 4T1 breast cancer and 3LL Lewis Lung carcinoma^{489,521}. Furthermore, the expression of IL4R α is observed on distinct TAM populations. For example, the expression of IL4R α is higher in Tie2⁺ myeloid cells compared to Tie2⁻ cells, and are part of a unique gene signature of the Tie2⁺ cells⁵²¹. Notably, the expression of IL4 is associated with MHCII^{low} TAMs as observed in our MSC-educated macrophages⁴⁸⁹.

Within our model of breast cancer bone metastasis, IL4R α is upregulated in BoMAMs, and is coexpressed with CD204. Furthermore, myeloid-specific IL4R α deficiency significantly reduces bone metastatic growth supportive of a functional role. We demonstrate that MSCs promote IL4R α expression in macrophages, and thus MSCs may indirectly promote bone metastatic growth by enhancing the expression of IL4R α in the macrophages.

It is now established that activation of TAM-expressed IL4R α through interaction with IL4 within the TME induces an M2-like TAM phenotype and promotes tumour growth and progression. In a model of breast cancer using the MMTV-PyMT model, CD4⁺ T cells are a major source of IL4, as well as other Th2 cytokines, including IL10 and IL13. This indirectly promotes tumour cell invasion and therefore metastasis to the lung by regulating the phenotype and function of TAMs, including the induction of an M2-like activation. Furthermore, IL4R α activation augments the induction of epidermal growth factor (EGF) in TAMs, which contributes to tumour cell invasion. Targeting of IL4R α using transgenic PyMT/IL4R α ^{-/-} mice reversed these effects attenuating metastasis to the lung⁵¹⁹. Interestingly, breast cancer bone metastasis in this project share a similar MMTV-PyMT origin and therefore may display similar effects. Similarly in a pancreatic cancer model, IL4 predominantly derived from cancer cells induces release of cathepsin B and S from TAMs, which promotes tumour growth, angiogenesis and invasion⁵²⁰. IL4 is also upregulated in several human cancers including breast, colon, pancreatic, ovarian and lung cancer where it has been shown to increase cancer cell

proliferation, promote resistance to apoptosis, enhance survival and maintenance of CSCs, and induce immunosuppressive effects on T cells^{522–526}. Together, this demonstrates a tumour-promoting role of IL4-IL4R interactions in cancer.

4.3.4.5 CD204

CD204, also known as scavenger receptor A (SR-A), is a member of the transmembrane receptors collectively termed as scavenger receptors that are expressed predominantly on macrophages and dendritic cells. CD204 is capable of binding a range of ligands and is involved in many processes including suppression of the inflammatory response^{527–529} and acts as an inhibitory receptor on activated macrophages⁵²⁷.

There is abundant evidence suggesting CD204⁺ macrophages play a substantial role in promoting tumour growth in several cancer types. The expression of CD204 on TAMs is correlated with tumour stage, relapse, and poor survival and prognosis in many cancers including lung cancer, breast cancer, kidney cancer, glioma, pancreatic cancer, bladder cancer and oesophageal cancer^{530–537}. CD204⁺ TAMs are enriched in the invasive front of pancreatic cancer, and is associated with poor prognosis and shortened survival as a result of accelerated lymphatic and liver metastasis. Abundance of CD204⁺ TAMs was also correlated to tumour size and early recurrence both locally and in the liver^{536,538}. CD204⁺ TAMs also promote ovarian cancer progression. Targeted depletion of CD204⁺ cells using saporin toxin conjugated with anti-CD204 monoclonal antibody in ovarian cancer also significantly reduced ovarian cancer progression and occurrence in the peritoneum⁵³⁹.

CD204 expression, in combination with CD163, are now considered as markers for pro-tumoural, M2-like TAMs, which display pro-angiogenic and immunosuppressive effects to promote tumour growth⁵³¹. CD204⁺ TAMs exert their pro-tumorigenic effects and contribute to tumour progression by regulation of the T cell response and angiogenesis. In a model of oral squamous cell carcinoma, CD163⁺ CD204⁺ TAMs were located in and around tumours in human samples, and was correlated with distant metastasis and reduced 5-year progression free survival. Flow cytometric analysis demonstrated that these CD163⁺ CD204⁺ TAMs produced IL10 and PDL1 at a higher level compared to CD163⁻ CD204⁺ CD163⁺ CD204⁻ TAMs. The co-culture of CD163⁺ CD204⁺ TAMs with CD3⁺ T cells results in reduced activation. Together, this demonstrates that CD163⁺ CD204⁺ TAMs inhibit T-cell response via the secretion of

IL10 and PDL1 creating an immunosuppressive environment that permits tumour growth⁵⁴⁰. CD204⁺ TAMs is also correlated with tumour progression and poor prognosis in lung squamous cell carcinoma. The abundance of CD204⁺ TAMs is also positively correlated with vessel density and number of regulatory FOXP3⁺ T cells in an ovarian cancer model, suggestive of pro-angiogenic and immunosuppressive roles, respectively⁵⁴¹. Comparably, CD204⁺ TAMs exhibit increased expression of VEGF⁵⁴².

Several factors have been implicated in the regulation of CD204-expression on TAMs, including IL10 and CCL2⁵³³. The expression of CCL2 in lung carcinoma was associated with increased CD204⁺ TAMs in the TME, supporting a role in recruitment⁵⁴¹. TNF α is also involved in the regulation of CD204 expression on TAMs in ovarian cancer. The co-culture of tumour cell with macrophages from mice deficient in TNF α -or its receptors resulted in reduced CD204 expression in comparison to culture with wildtype control macrophages. Furthermore, TAMs from ovarian cancer treated with neutralising TNF α antibodies or those established TNF α -deficient mice exhibited less CD204 expression⁵⁴².

CD204 is also upregulated in BoMAMs, and is co-expressed with both IL4R and Tim4. The activation of IL4R α with IL4 promotes CD204 expression as well as other M2-associated markers on TAMs^{519,520} and may therefore provide a reason for their co-expression. We also demonstrate that MSCs induce increase IL4R α in macrophages, which is in agreement with previous studies that demonstrate MSC-mediated induction of CD204 as part of the M2 signature of TAMs⁴²².

4.3.4.6 Summary of MSC-educated macrophages

Overall, the expression pattern of the MSC-educated macrophages is similar to the overall M2-like TAM phenotype that we also observe in BoMAMs *in vivo*, particularly for PD1, IL4R, CD204 and MHCII. These BoMAMs display an immunosuppressive, pro-angiogenic phenotype to promote tumour growth. In addition, each surface molecule may also play a specific role in promoting tumour growth and development as described above and summarised in table 4.3.2.

In summary, we propose that MSCs recruit macrophages toward the bone metastatic region, where they induce polarisation into a TAM-like phenotype, similar to that observed in BoMAMs. This subsequently promotes bone metastasis growth as described by Ma *et al.* (submitted). It is clear that a interactions exists between the

TAMs and MSC/CAFs, and is further complicated by the role of the tumour cells in activating the MSC/CAFs or macrophages. Further investigation is required to confirm the MSC-macrophage interactions *in vivo* and to identify the molecular mechanisms involved, which may be inhibited to disrupt the tumour-promoting effects.

Marker	Factors regulating expression	Pro-tumorigenic effects of marker
PD1	IFN γ , TLF2/4	•Osteoclastogenesis •T cell suppression •Inhibition of phagocytosis •Inhibition of immune recognitions
PDL1	TNF γ , IFN γ , IL4, IL6, IL10, IL10, IL27, IL13, CXCL8, exosomes, OPN, chitin, PKM2, COX2, PGE2	•T cell suppression •Macrophage proliferation •IL12, IL1 β, CCL2, CCL7, CXCL3, TNFα
IL4Rα		•Cathepsin B + S $\rightarrow$ angiogenesis, growth and invasion •TNFα, CCL2, CCL9, CCL17, EGF •CSC formation •Cancer cell proliferation •Apoptosis resistance •T cell suppression •M2 marker expression
CD204	IL10, CCL2, TNF α	•IL10 $\rightarrow$ T cell suppression •VEGF $\rightarrow$ angiogenesis
Tim4	DAMPs, TGF β , IL6, TNF α	•T cell suppression via uptake of apoptotic cells •CSC formation •EMT and invasion •Angiogenesis
MHCII	FOXO1, hypoxia	•MMP9 $\rightarrow$ invasion •IL10, TGF β, VEGF, TNFγ •$\downarrow$ CCL5, CX3CL1, CXCL11, CXCL10, CCL17, CCL22 $\rightarrow$ $\downarrow$ T cell recruitment •CCL6, CCL7, CCL12, CCL4, CCL3, CCL9 $\rightarrow$ monocyte recruitment •T cell suppression •Angiogenesis

Table 4.3.2: Roles of BoMAM-markers in promoting tumour progression.

4.3.5 Role of OBs

Osteoblasts are a pivotal player in the 'vicious cycle' of bone metastasis growth⁵⁴. We therefore used an osteoblast depletion model to determine the role of osteoblasts in breast cancer bone metastasis. We hypothesise that osteoblast depletion would disrupt this growth-promoting osteolytic cycle in bone metastasis. In contrast, it was demonstrated that osteoblast depletion has no significant effect on bone metastasis. This is complemented with *in vitro* assays where the co-culture of bone metastatic breast cancer cells with osteoblasts does not significantly affect tumour growth. Although a larger sample size is required to confirm these results, this preliminary data suggests that osteoblasts do not play a significant role in the growth stage of breast cancer bone metastasis.

Our data is supported by a previous study by Zheng *et al.*⁵⁴³ who demonstrate that chemotherapy-treated osteoblasts upregulate Jagged1 to promote survival of breast cancer cells in the bone, whereas osteoblasts do not contribute to growth in untreated bone metastasis, although the effect of osteoblast depletion was not studied⁵⁴³. Conversely, another study demonstrates that tumour-derived Jagged1 promotes breast cancer bone metastatic growth by stimulating the release of IL6 from osteoblasts via activation of the Notch pathway. Osteoblast-derived IL6, in turn, activates osteoclastogenesis that promotes osteolysis and further formation of an osteolytic lesion. In addition, TGF β is released during osteolysis, which acts upstream of Jagged1 to promote further release. This creates a growth-promoting paracrine loop comprised of tumour-derived Jagged1, osteoblast-derived IL6 and osteoclast-mediated release of TGF β , which can be disrupted by inhibition of the Notch pathway using γ -secretase inhibitor⁵⁴⁴. Interestingly, both elevated Jagged1 and Notch1 expression is significantly associated with poor prognosis of patients with breast cancer⁵⁴⁵. These different effects of Jagged1 in these studies may be accounted for by the different cell sources.

Hughes *et al.*⁵⁴⁶ also demonstrated that osteoblast expansion as a result of zoledronate treatment, which is an anti-osteoclast drug used to prevent bone loss, is correlated with reduction in breast cancer metastasis growth. This therefore suggests that osteoblasts may play an inhibitory role in bone metastatic growth. However, other cells are affected by zoledronic acid and therefore they do not show the direct effects of osteoblast only depletion on growth⁵⁴⁶.

Lastly, it was also previously shown that specifically senescent osteoblasts promote tumour cell seeding and growth. These senescent osteoblasts contribute to bone metastatic growth by secreting IL6, which promotes osteoclastogenesis⁹⁰. It may be that distinct populations of osteoblast play different roles and that complete depletion of all populations counteracts these effects resulting in no overall change.

Furthermore, breast cancer bone metastasis exhibits an osteolytic phenotype, whereas bone metastasis of other cancers such as prostate cancer is osteoblastic. Osteoblasts may therefore play a more significant role in prostate cancer bone metastasis, whilst breast cancer bone metastasis is less affected. Due to the limited response of osteoblast depletion, we propose that the total osteoblast population does not contribute to the breast cancer metastatic growth.

4.4 Conclusion

Whilst it is established that mesenchymal stromal cells significantly contribute to tumour growth in several primary cancer models, research into their role at secondary sites, particularly bone metastasis, is limited. Here, through the use of *in vivo* depletion models, we demonstrate that mesenchymal cells, particularly nestin⁺ MSCs, play a pivotal role in the growth and survival of breast cancer bone metastasis. This is supported by *in vitro* proliferation assays that determine MSCs promote bone metastatic growth in a MSC number-dependent manner. Moreover, we provide evidence that soluble factors secreted from bone metastatic cells promote both proliferation and recruitment of MSCs in breast cancer bone metastasis therefore establishing a potential paracrine loop. We further show that other subtypes of mesenchymal cells, including CD146⁺ MSCs and fibroblasts, may also exhibit tumour-promoting effects in bone metastasis, and it is key that these cells are further characterised in bone metastasis. Our data suggests that osteoblasts do not appear to contribute to breast cancer bone metastasis, although this requires further experiments to validate the results.

We next explored potential mechanisms involved in MSC-mediated metastasis growth, as summarised in figure 4.4.1. First we demonstrate using *in vitro* assays that MSCs secrete growth-promoting factors that act directly on tumour cells and is dependent on direct contact between the MSCs and tumour cells. Additional experiments are required to identify the potential molecular mechanisms involved. Secondly, we provide preliminary data to show that MSCs may undergo differentiation induced by the bone metastatic cancer cells to give rise to tumour-promoting CAFs, which were also identified in bone metastasis. We propose a model where direct contact between the MSCs and tumour cells induces differentiation towards a CAF-like phenotype, which subsequently produce soluble factors that promote bone metastatic growth. This hypothesis needs to be tested *in vivo* using lineage tracing experiments. Alternatively, MSCs may also exert their pro-tumorigenic effects via modulation of the immune system, particularly the macrophages. We therefore performed pilot experiments that show that MSCs promote migration of macrophages and induce a M2-like macrophage phenotype. This is similar to the phenotype observed in BoMAMs, which are defined as a tumour-promoting population of macrophages in breast cancer bone metastasis in another project. We therefore propose that MSCs may also promote bone metastatic growth indirectly by promoting macrophage recruitment and subsequent polarisation

into an immunosuppressive, pro-angiogenic phenotype, which permits tumour growth. Future experiments, including the characterisation of the macrophages in nestin⁺ MSC-depleted mice described above are required to confirm this mechanism *in vivo*.

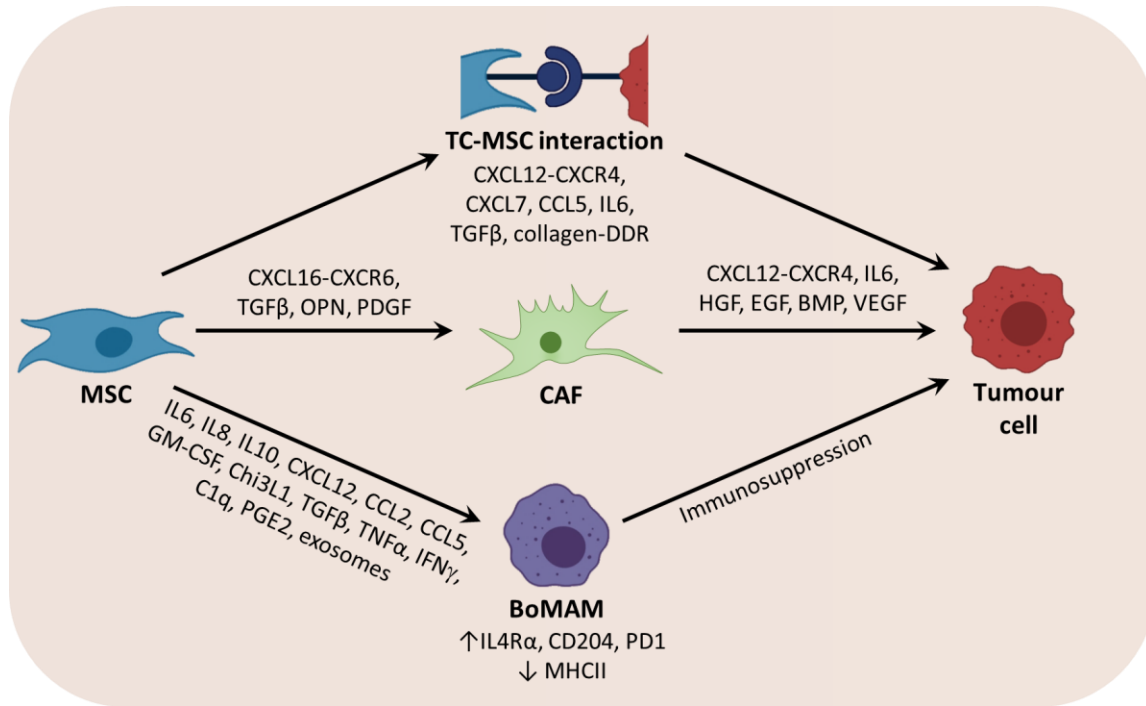


Figure 4.4.1: Potential mechanisms involved in MSC-mediated bone metastasis growth and previous molecular interactions implicated in these processes. MSCs can secrete molecular factors that directly interact with tumours to promote growth. MSCs also undergo differentiation to give rise to tumour-promoting CAFs, which is dependent on tumour-derived factors. MSCs also interact with other components of the TME, particularly macrophages, to induce a TAM-like immunosuppressive phenotype.

To our knowledge, this is the first study that demonstrates a role of nestin⁺ MSCs in the growth of breast cancer bone metastasis. Due to the substantial effect of nestin⁺ MSC depletion on breast cancer bone metastatic growth and the tumour-promoting effects of MSCs in *in vitro* cultures, we propose a therapeutic benefit of targeting these cells. It is therefore important to determine the molecular interactions involved in the paracrine loops that exist between the tumour cells, the mesenchymal compartment and other cells within the TME as disruption of these interactions may offer insight into potential therapeutic targets for treatments of established bone metastasis.

Chapter 5 Effect of chemotherapy treatment on breast cancer bone metastasis

5.1 Introduction

Chemotherapy is an integral component of breast cancer treatment in both primary and recurrent tumours. Current standard of care for regional disease often includes chemotherapy as a first line therapy used either alone, or in a neoadjuvant or adjuvant setting in conjunction with surgery and/or radiotherapy. The anti-cancer effect of chemotherapy is developed around the concept of killing or halting the growth of cancer. The mechanisms used to achieve this vary between chemotherapy types, but ultimately result in the induction of cell death, and thus eradication of the cancer cells from the body.

Whilst the majority of primary tumours respond well to chemotherapy resulting in successful elimination of the cancer, relapse can occur in a proportion of the patients emerging either at the original location of the primary tumour or at the metastatic site. Late stage disease characterised by recurrence at the local site or metastasis displays resistance, and remains one of the major challenges in clinical oncology, accounting for over 90% of cancer-associated deaths.

Chemotherapy resistance was previously attributed to several cell autonomous processes that underlie clinical drug resistance, and were acquired during treatment. This includes expression of efflux pumps and drug transporters such as p-glycoprotein, multidrug resistance protein (MRP1) and breast cancer resistant protein (BCRP); alterations in DNA repair pathways; acquisition of secondary mutations in drug targets; constitutive activation of signalling pathways; and the selection of a drug resistant clone^{95,547,548}. Much of this research was performed in *in vitro* experiments on human cell lines and in the last few decades research has recognised that the TME plays a key role in resistance. Chemotherapy induces infiltration and activation of components within the TME that subsequently promote resistance via the secretion of molecular factors. This includes recruitment and activation of the immune and mesenchymal compartment, which exert tumour-promoting effects such as immunosuppression and

neovascularisation^{549,550}. This research has predominantly focussed on resistance within primary tumours, the contribution of the TME at secondary sites whilst secondary sites, particularly in bone metastasis, remains to be elucidated. Bone metastasis is often treated using chemotherapy as a palliative therapy in the aim to constrain the current size or restrict further growth in order to prevent osteolysis. However, existing lesions usually continue to progress whilst new lesions develop, and eventually patients unfortunately succumb to the disease.

We will therefore utilise our *in vivo* model to investigate the effects of chemotherapy in breast cancer bone metastasis (figure 5.1.1). We also aim to identify the changes within the TME that are responsible for the chemotherapy resistance observed in the clinic with a particular focus on two commonly used chemotherapy types: paclitaxel (PTX), which is a taxol-based drug that stabilises the microtubules during mitosis and therefore inhibits cell division; and doxorubicin (DOX), which is an anthracycline that inhibits progression of topoisomerase II enzymes along the DNA therefore prevents cell division and induces cell death due to interruption of DNA replication. Both chemotherapies are a component of the second-line treatment strategy of secondary breast cancer. However, paclitaxel and doxorubicin exhibit low response rates of 15-40% and 15-30% with median survival durations of 15.6 and 18.3 months, respectively⁵⁵¹. The identification of the processes leading to the resistance may therefore provide insight into targeted therapies that prevent relapse.

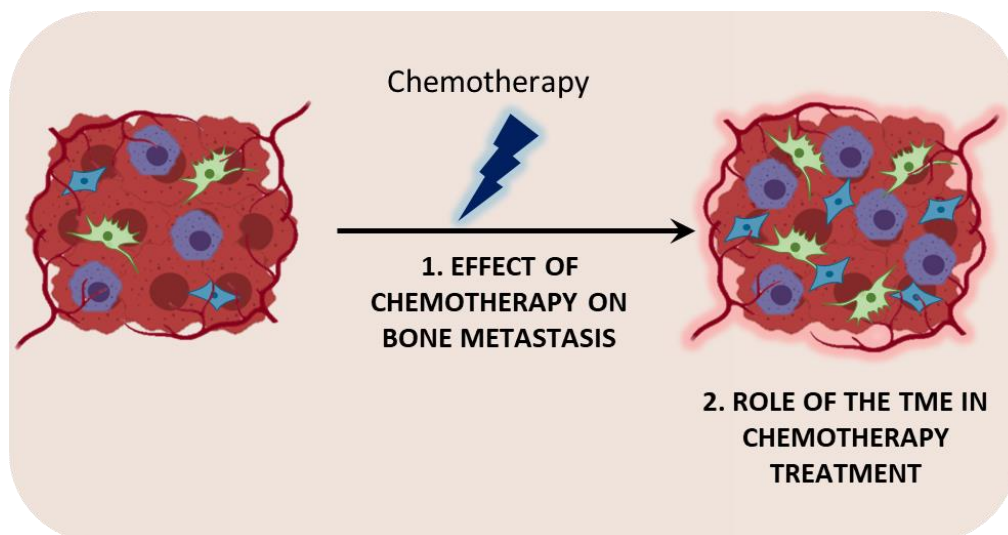


Figure 5.1.1: Effect of chemotherapy on bone metastasis and the role of the TME in the response.

5.2 Results

5.2.1 Bone metastasis response to chemotherapy

To study the effects of chemotherapy in bone metastasis, we utilised a standard chemotherapy treatment regime in the bone metastatic model (figure 5.2.1a). Breast cancer bone metastasis was established in FVB/N mice via intracardiac injection of MetBo2 cells. Once tumours were detected, mice were treated with PTX (10mg/kg) or DOX (5mg/kg) via intraperitoneal injection every 5 days. This is a standard chemotherapy regime previously utilised in the MMTV-PyMT-based breast cancer models where the dosages and frequency of treatment have been optimised and translate to a clinically relevant concentration^{552–554}. Mice were sacrificed on day 15 of treatment and tissues collected for analysis. Bioluminescence imaging was used to monitor tumour growth throughout the treatment regime. The signal was normalised to day 0 of treatment to allow comparison between untreated and PTX- or DOX-treated bone metastasis.

DOX-treated metastasis progressed at a similar rate to untreated (UNT) metastasis with no significant difference of tumour size on day 15. Whilst there is a slight increased growth during the early stage of growth in DOX-treated metastasis, this was non-significant. In contrast, PTX-treated metastases exhibit increased growth compared to untreated metastases. This is particularly prominent in the later stages with almost a six-fold increase in tumour size on day 15 (figure 5.2.1b. figure 5.2.1c). The effect of chemotherapy treatment on survival was also measured as the time from intracardiac injection to humane endpoint. Neither chemotherapy type had any significant effect on survival with a slight trend towards reduced survival in chemotherapy-treated mice (figure 5.2.1d). This suggests that bone metastasis is resistant to DOX, whilst PTX induces growth, defined as chemotherapy-induced metastatic growth.

To investigate the role of the bone microenvironment in resistance and chemotherapy-induced growth, we tested the chemotherapy response of MetBo2-derived tumours in mammary fat pad (MFP) (figure 5.2.2a). Tumours were established via percutaneous injection into the MFP and the same chemotherapy treatment regime was initiated once tumours became palpable. Tumour growth was monitored using calliper measurements to calculate tumour volume, and normalised to day 0 of treatment. Whilst untreated tumours grew to 6-times of the original size, DOX- and PTX-treated tumours only grew

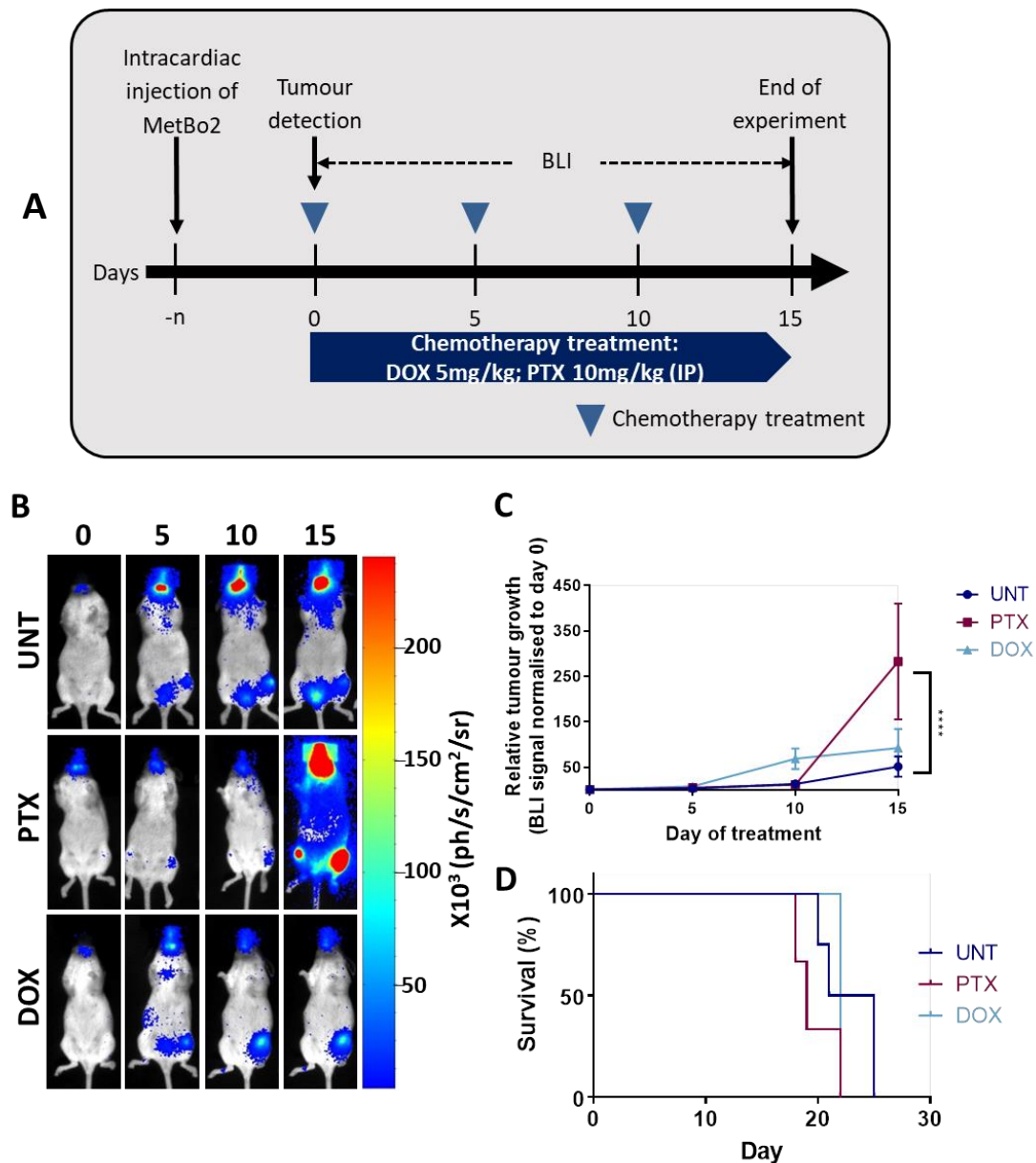


Figure 5.2.1: Bone metastasis are resistance to doxorubicin and paclitaxel promotes bone metastatic growth. A) Experimental design: chemotherapy treatment in MetBo2-derived experimental bone metastasis in FVB/N mice (IP, intraperitoneal; BLI, bioluminescence imaging). **B)** Representative images of the BLI signal of each chemotherapy group on days 0, 5, 10 and 15. **C)** Quantification of bone metastatic growth throughout chemotherapy treatment. Growth was monitored using bioluminescent imaging on day 0, 5, 10 and 15. Data shown are mean $\pm$ SEM (**** P <0.0001, two-way ANOVA, n =12-19). **D)** Survival of mice during chemotherapy treatment measured as the number of days from intracardiac injection to humane endpoint. (n =12-19).

to approximately half this size (figure 5.2.2b). Due to issues with ulceration in all groups, the treatment could not be continued beyond 5 days of treatment. Tumour cells were also injected with collagen in effort to prevent the ulceration, but similar issues occurred. Mice were therefore sacrificed and tumours collected for analysis at day 5. This resulted in a relatively short therapeutic window between tumour detection and

humane end point, and therefore could only be treated for 5 days compared to 15 days of treatment in bone metastasis. Nevertheless, both types of chemotherapy treatment significantly reduced tumour growth during this time period, and exhibited a similar response to other MMTV-PyMT-based models^{552,554}. In contrast to bone metastasis, MetBo2-derived tumours established in the MFP were sensitive to chemotherapy treatment even after only 5 days of treatment, which suggests that the effects of chemotherapy were specific to the bone metastasis. It also confirms that the chemotherapy is effective and the response observed, particularly in DOX-treated mice, were not due to issues with the chemotherapy treatment regime.

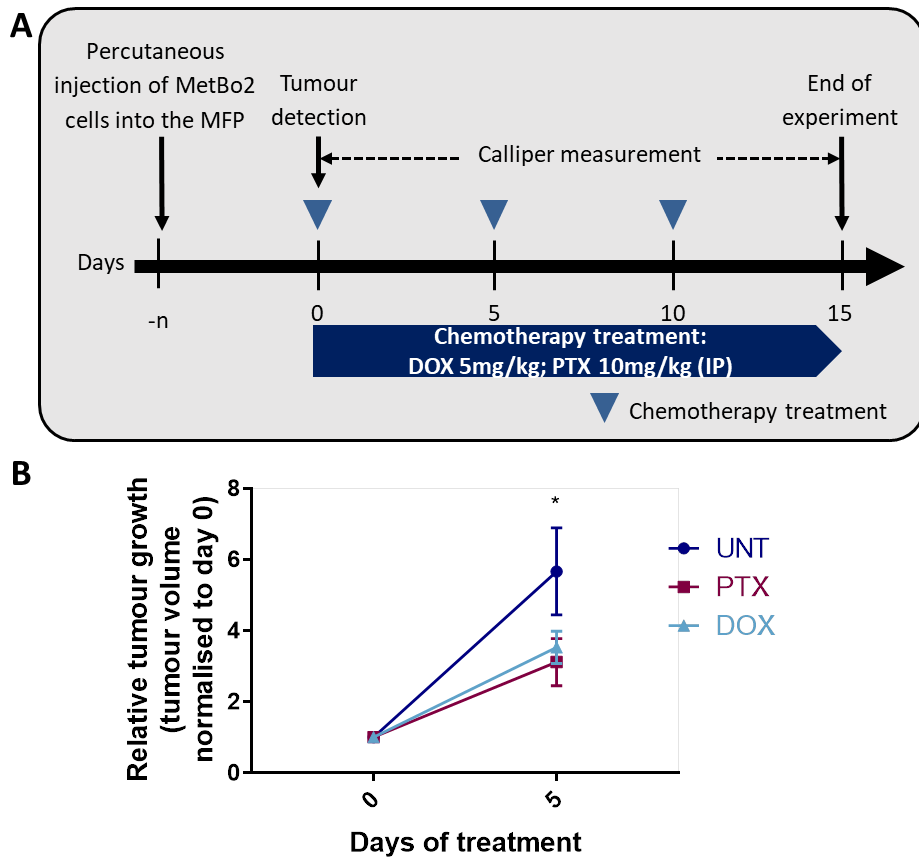


Figure 5.2.2: Tumours in the MFP are sensitive to chemotherapy and respond to paclitaxel and doxorubicin treatment. **A)** Experimental design: chemotherapy treatment of MetBo2-derived orthotopic tumours in the MFP of FVB mice (MFP, mammary fat pad; IP, intraperitoneal). **B)** Quantification of MetBo2-derived MFP tumour growth throughout chemotherapy treatment. Growth was monitored using calliper measurements of the tumour to calculate the tumour volume. Data shown are mean $\pm$ SEM (* p <0.05 (UNT vs PTX – 0.0131, UNT vs DOX – 0.0345), two-way ANOVA, n =8-10).

To test the role of the TME within the bone metastasis, we recovered MetBo2 cells from untreated and chemotherapy-treated bones and tested their response to chemotherapy *in vitro*. MetBo2 cells were seeded into 24-well plates and treated PTX or DOX at a range of concentrations for 5 days, after which an MTT assay was performed to measure tumour cell number. In comparison to naïve, parental MetBo2 cells, MetBo2 cells recovered from PTX- and DOX-treated bone metastasis exhibited the same chemotherapy response with an IC₅₀ of 3nM and 3.5nM, respectively (figure 5.2.3). This suggests that the chemotherapy resistance and induced growth observed *in vivo* was due to the TME within the bone, rather than cell autonomous changes acquired during treatment.

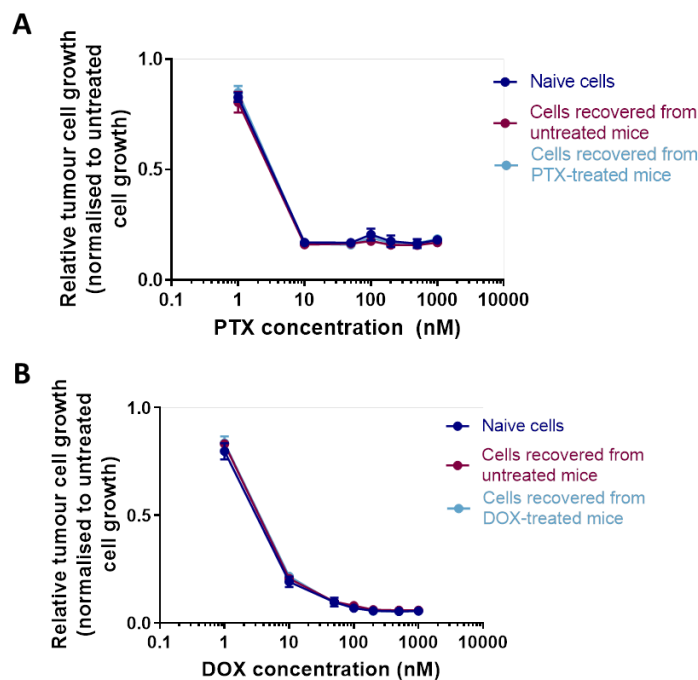


Figure 5.2.3: MetBo2 cells isolated from *in vivo* untreated and chemotherapy-treated bone metastasis recover their chemotherapy sensitivity *in vitro*. Growth of MetBo2 cells quantified by MTT assay on day 3 of **A)** paclitaxel or **B)** doxorubicin treatment. Data shown are mean $\pm$ SEM (n=3-4).

Chemotherapy exhibits a cytotoxic activity by inducing apoptosis and preventing proliferation of the cancer cells^{547,548}. We therefore analysed the abundance of apoptotic and proliferative cells in each tumour type using cleaved caspase (CC3) and Ki67 staining, respectively. PTX- and DOX-treated bone metastasis exhibited decreased apoptosis compared to untreated bone metastasis whilst apoptosis was increased in PTX- and DOX-treated MFP tumours (figure 5.2.4). Although this was not significant, it suggests that chemotherapy-treated bone metastasis is partly resistant to

apoptosis, whilst apoptosis is induced in MFP tumours. There is no significant difference in the number of proliferating cells in untreated, PTX- and DOX-treated bone metastasis, whereas proliferating cells are slightly decreased in PTX- and DOX-treated MFP compared to the untreated group (figure 5.2.5). Moreover, the number of proliferating cells in bone metastasis was significantly lower than the number of proliferating cells in the MFP in each treatment group.

Together, this data suggests that the TME within bone metastasis is responsible for DOX resistance and PTX-induced growth, and that this effect is specific to the bone. By comparing the apoptotic states of bone metastatic cells and MFP tumours in response to chemotherapy treatment, we demonstrate that environment of bone metastasis may have a protective effect against chemotherapy-induced apoptosis. Chemotherapy also reduces the number of proliferating tumour cells in MFP tumours, whereas no change was observed in PTX-treated bone metastasis with a slight increase in DOX-treated bone metastasis. Whilst there is no significant difference in the abundance of apoptotic and proliferating tumour cells between treatment groups, the combination of reduced apoptosis and unchanged proliferation in treated bone metastasis may account for the chemotherapy resistance and chemotherapy-induced growth. Furthermore, bone metastases also exhibit a significantly lower abundance of proliferating cells in comparison to the MFP tumours. This may contribute to the resistance as chemotherapy shows highest efficiency targeting proliferating bone metastatic cells whilst non-dividing cells are resistant^{75,555}.

5.2.2 Role of the immune compartment in chemotherapy-treated bone metastasis

We next investigated changes in TME induced during chemotherapy treatment to identify the potential cell population responsible for the effects of chemotherapy. We first analysed the immune compartment with a particular focus on the macrophages and lymphocytes. The abundance of cell populations was quantified using immunofluorescent staining and flow cytometry analysis of the immune cells in untreated and chemotherapy-treated bone metastasis. (Immunofluorescent staining was performed by Tanya Kapustsenka, Chengbin Zhang and Jivishini M, and analysed by myself). We also compared the effect of chemotherapy on the TME in bone metastasis and MFP tumours to give insight into potential differences accounting for the opposing chemotherapy efficiencies between the tumour types.

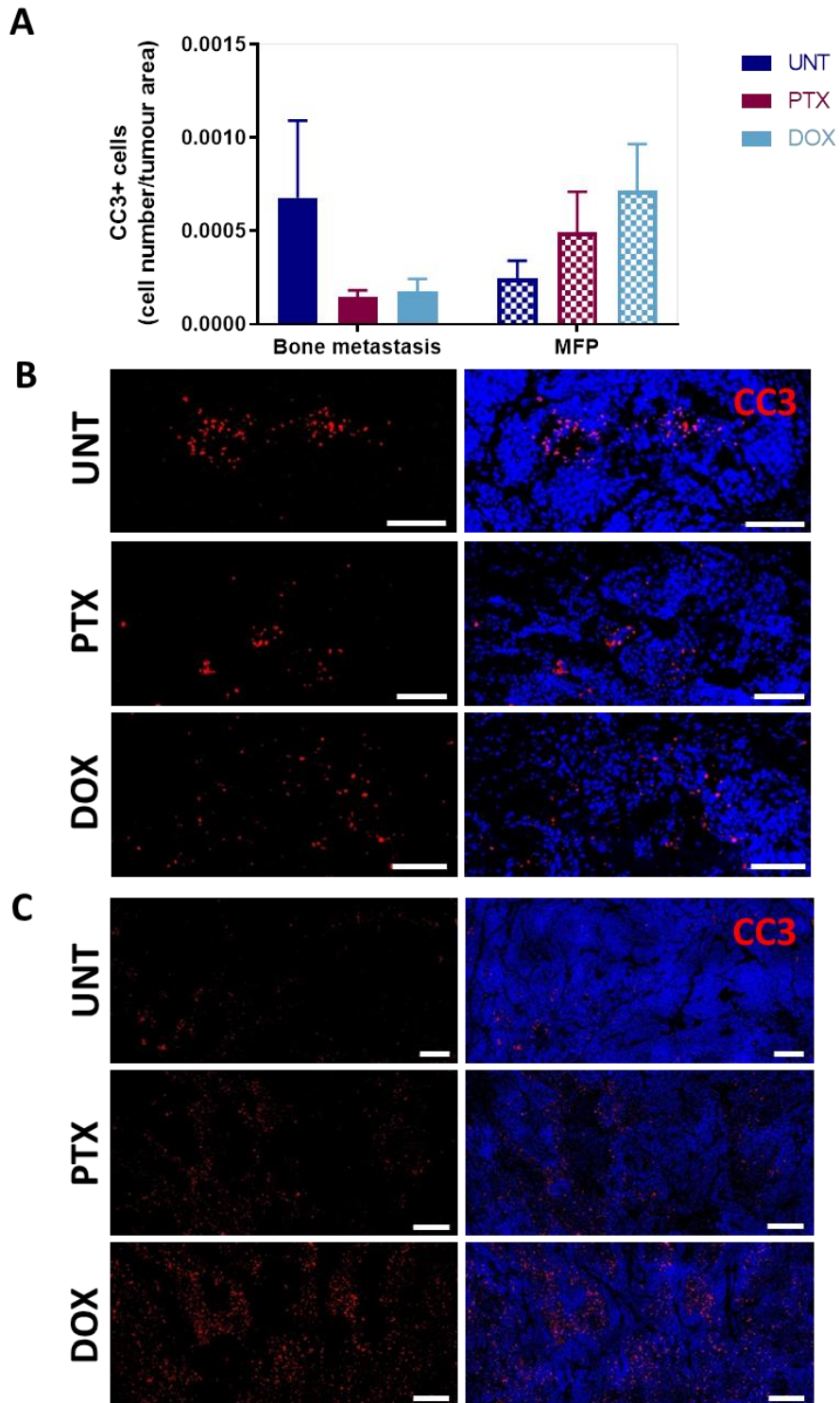


Figure 5.2.4: Bone metastasis appear resistant to apoptosis during chemotherapy treatment. **A)** Quantification of CC3+ cells in bone metastasis and MFP tumours on day 15 and day 5 of chemotherapy-treatment regime, respectively. Data shown are mean $\pm$ SEM (bone n=12-14, MFP n=3-8). **B+C)** Representative immunofluorescence images stained for apoptotic marker CC3 (left) and merged with DAPI (right) in untreated, PTX-treated and DOX-treated **B)** bone metastasis. Scale bar: 100 μ m and **C)** MFP tumours. Scale bar: 200 μ m.

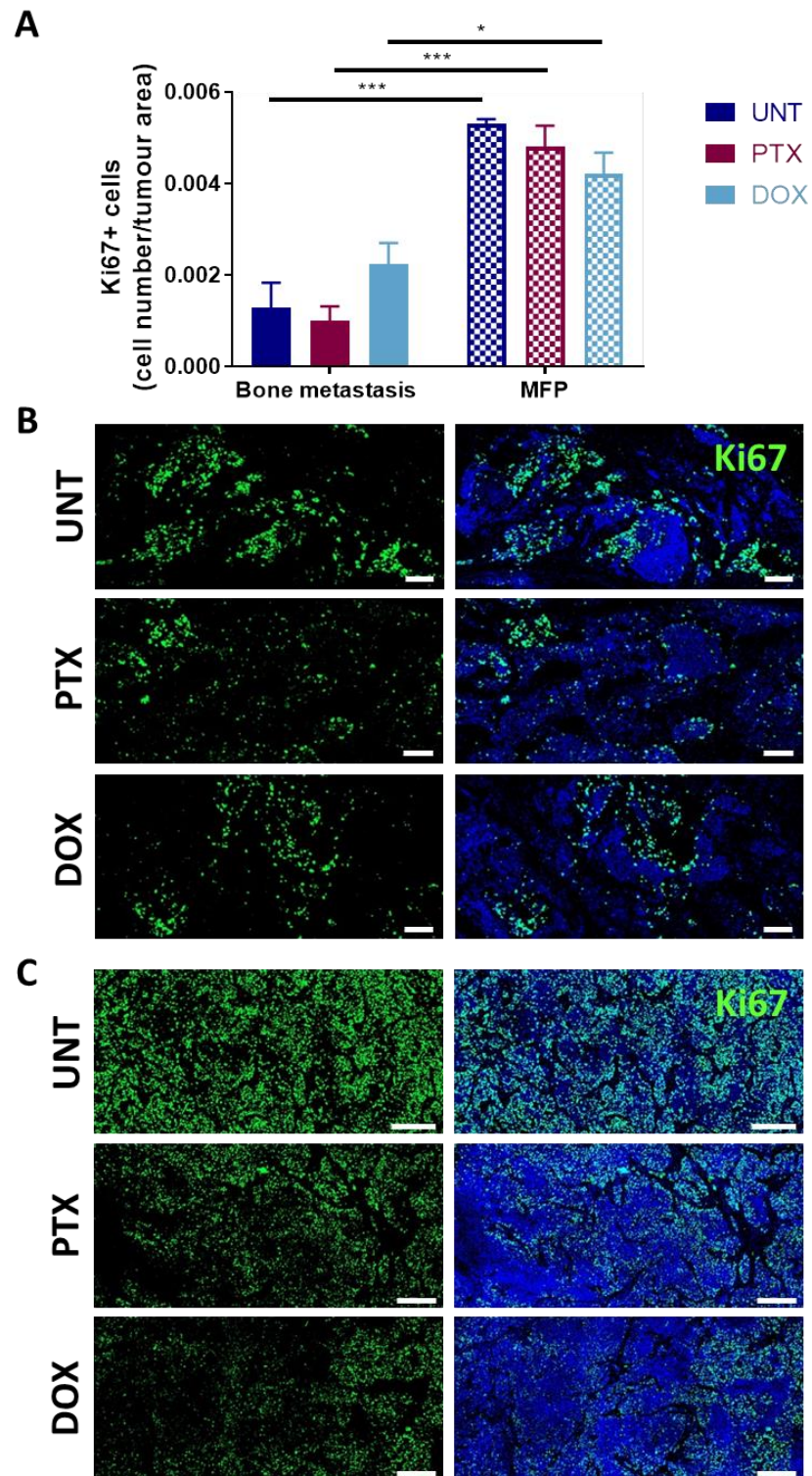


Figure 5.2.5: Bone metastasis exhibits significantly reduced proliferation compared to MFP tumours and is unaffected by chemotherapy treatment. **A)** Quantification of Ki67+ cells in bone metastasis and MFP tumours on day 15 and day 5 of chemotherapy-treatment regime, respectively. Data shown are mean $\pm$ SEM (* p <0.05 *** p <0.0005, two-way ANOVA, bone n =7-8, MFP n =3-8). **B+C)** Representative immunofluorescence images stained for proliferation marker Ki67 (left) and merged with DAPI (right) in untreated, PTX-treated and DOX-treated **B)** bone metastasis and **C)** MFP tumours. Scale bar: 100 μ m.

5.2.2.1 Myeloid cells

Monocytes and macrophages have previously been shown to contribute to chemotherapy resistance¹⁷⁷ and chemotherapy-induced metastasis⁵⁵⁶, and play a significant role in bone metastatic growth (Ma *et al.* submitted). The effect of chemotherapy treatment on macrophage abundance was analysed using immunofluorescent staining of Iba1 in bone metastasis and in MFP tumours (figure 5.2.6). Whilst chemotherapy treatment induces a slight increase in macrophage abundance in bone metastasis, there is no significant difference. In contrast, treatment of both chemotherapies reduces macrophage abundance in MFP tumours compared to untreated tumours. However, this is not significant due to variation in untreated tumours. As the variation in macrophage abundance may be due to the difference in tumour size or response to chemotherapy, we analysed the correlation between Iba1 abundance and BLI signal or MFP tumour volume as well as the number of Ki67⁺ proliferating cells. No correlation was observed in any treatment group (data not shown). Nevertheless, this data suggests that the macrophage response to chemotherapy-treatment may be different between the TME of bone metastasis and MFP.

Previous studies that analysed the effects of TAMs in chemotherapy response demonstrated that the paclitaxel induced significant changes in the intratumoral myeloid cells during the first 24-72 hours after chemotherapy administration and before significant difference in tumour volume was observed^{143,557}. We therefore performed a detailed analysis of the populations in the myeloid lineage using flow cytometry. Bones were collected 24 hours after chemotherapy treatment, separated into the tumour-enriched and non-enriched regions, and digested to a single cell suspension for staining. Cells were stained with CD11b, Ly6C, Ly6G, CD115, CCR2, F4/80 and CD204 to identify the following cells in the myeloid panel: neutrophils (CD11b⁺ Ly6G⁺ Ly6^{int}), monocytes (CD11b⁺ Ly6G⁻ Ly6C⁺ CD115⁺ CCR2^{+/-}) and macrophages (CD11b⁺ Ly6C⁻ Ly6G⁻ F4/80⁺ CD204^{+/-}) (figure 5.2.7). Analysis of the macrophage population confirms that there is no significant difference between total macrophages in untreated and PTX-treated bone metastasis after short term chemotherapy treatment (figure 5.2.8a). CD204⁺ macrophages, which are an important subpopulation of macrophages in untreated breast cancer bone metastasis (Ma *et al.* submitted), were also unchanged in PTX-treated bone metastasis compared to untreated bone metastasis. However,

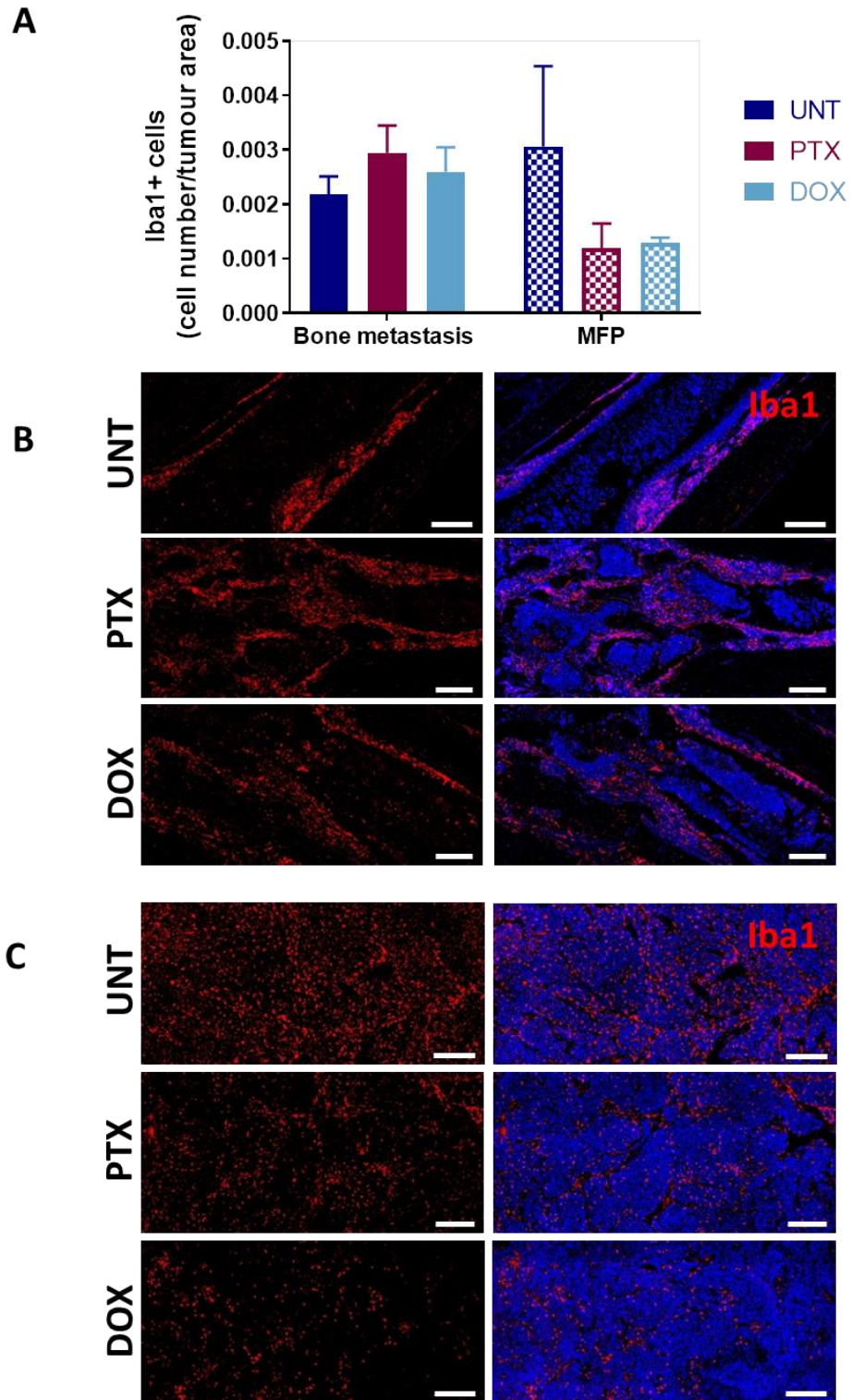


Figure 5.2.6: Chemotherapy-treated bone metastasis exhibit a slight increase in macrophages compared to chemotherapy-treated MFP tumours, where macrophages are reduced. **A)** Quantification of Iba1+ cells in bone metastasis and MFP tumours on day 15 and day 5 of chemotherapy-treatment regime, respectively. Data shown are mean $\pm$ SEM (bone n=5-12 MFP n=3-4). **B+C)** Representative immunofluorescence images stained for macrophage marker Iba1 (left) and merged with DAPI (right) in untreated, PTX-treated and DOX-treated **B)** bone metastasis and **C)** MFP tumours. Scale bar: 200 μ m.

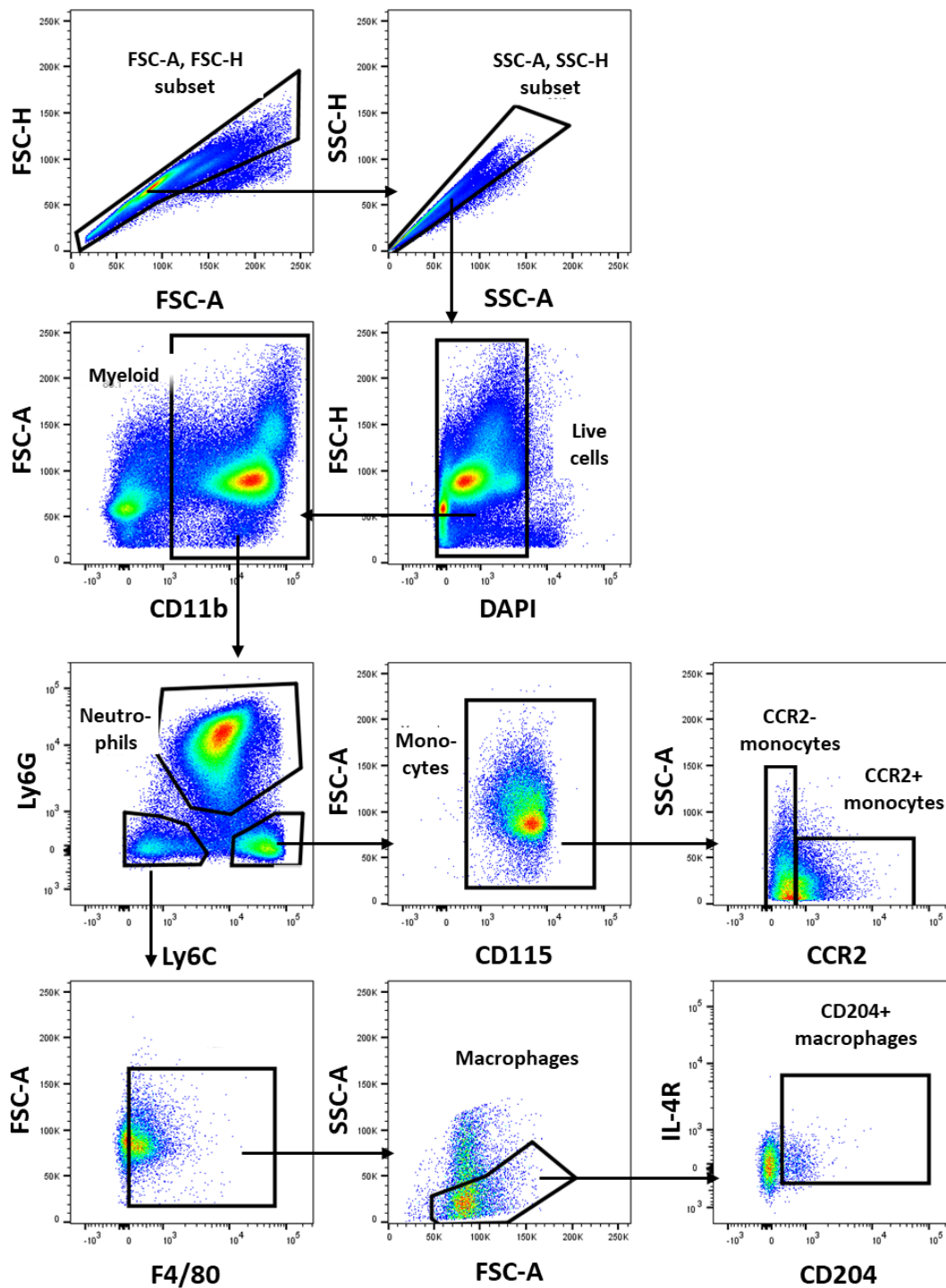


Figure 5.2.7: Representative gating strategy of the myeloid lineage in bone metastasis.

in agreement with previous results, CD204⁺ macrophages were slightly enriched in the tumour region of both treatment groups. We also demonstrate that there were no significant differences in the abundance of total monocytes, CCR2⁺ monocytes and CCR2⁻ monocyte populations in the tumour regions of untreated and PTX-treated bone metastasis (figure 5.2.8b).

To determine any functional role of macrophages in chemotherapy response of bone metastasis, we determined the effect of macrophage depletion during chemotherapy treatment. We first utilised the well-established macrophage depletion method of treating mice with liposome-encapsulated clodronate (liposome clodronate) as previously described⁵⁵⁸. Previous studies also demonstrate that liposomes are not taken up by PyMT-derived or Met1 tumour cells^{558,559}. Bone metastases were treated with chemotherapy as before and liposome clodronate was administered via intravenous injection twice/week during the chemotherapy treatment (figure 5.2.9a). Due to significant adverse effects displayed by the mice between day 5 and 8 of chemotherapy treatment, the experiment had to be terminated early. A similar response to the previous non-depleted mice was observed in each chemotherapy group during this early stage of treatment (figure 5.2.9b).

Liposome clodronate also depletes other cells, including osteoclasts, osteoblasts and osteoprogenitor cells⁵⁶⁰, which may account for the adverse effects observed. We therefore tested an alternative model of macrophage depletion using a CSF1R inhibitor, BLZ-945 (BLZ). BLZ selectively binds to CSF1R expressed on macrophages and inhibits proliferation of resulting in macrophage depletion. We also only focussed on PTX treatment as the protocol was being optimised. Mice were administered with BLZ (200mg/kg) daily via oral gavage during the chemotherapy treatment regime. Non-depleted controls were administered with vehicle only (figure 5.2.10a). Whilst the experiment again had to be terminated between day 7-10 due to adverse effects, no difference in chemotherapy response was observed between macrophage depleted bone metastasis and non-depleted controls (figure 5.2.10b). This data suggests that macrophages do not play a significant role in the chemotherapy response of bone metastasis during the first 7 days of the treatment regime.

We also utilised the CD169-DTR transgenic mice to explore the role of CD169⁺ bone resident macrophages in the chemotherapy response (figure 5.2.11a). It should be noted that this experiment was performed by Ruoyu Ma. The administration of DT in

the CD169-DTR mice induces depletion of the CD169⁺ macrophages. Non-depleted controls were administered with mutated Glu⁵² DT. A similar chemotherapy response to both PTX and DOX was observed in these mice compared to previously used FVB/N mice. The depletion of CD169⁺ macrophages did not affect the response with identical growth rates observed in both non-depleted and depleted mice in each chemotherapy group (figure 5.2.11b). Although the experiment had to be terminated on day 10 due to adverse effects, it provides evidence that CD169⁺ bone resident macrophages do not play a role in the chemotherapy response of bone metastasis.

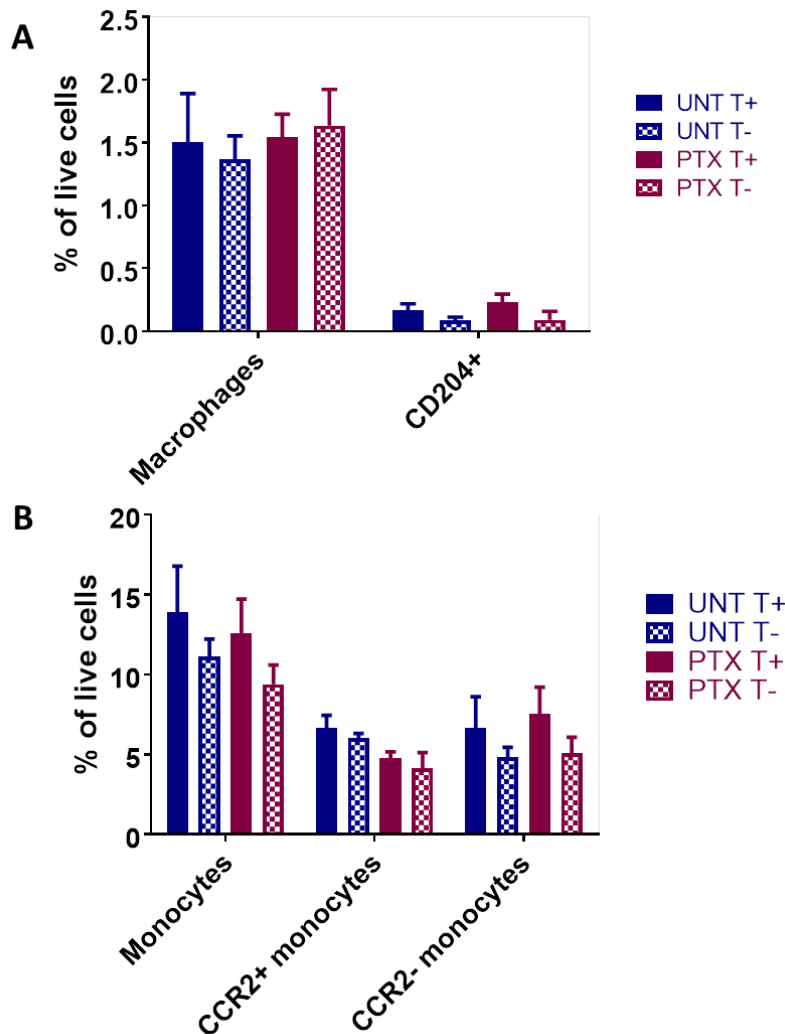


Figure 5.2.8: Myeloid populations in bone metastasis are not significantly affected by paclitaxel treatment. Quantification of **A**) macrophages and **B**) monocytes populations on day 6 of the chemotherapy treatment regime measured using flow cytometry analysis. (T+, tumour enriched region; T-, tumour non-tumour region of bone) (n=4).

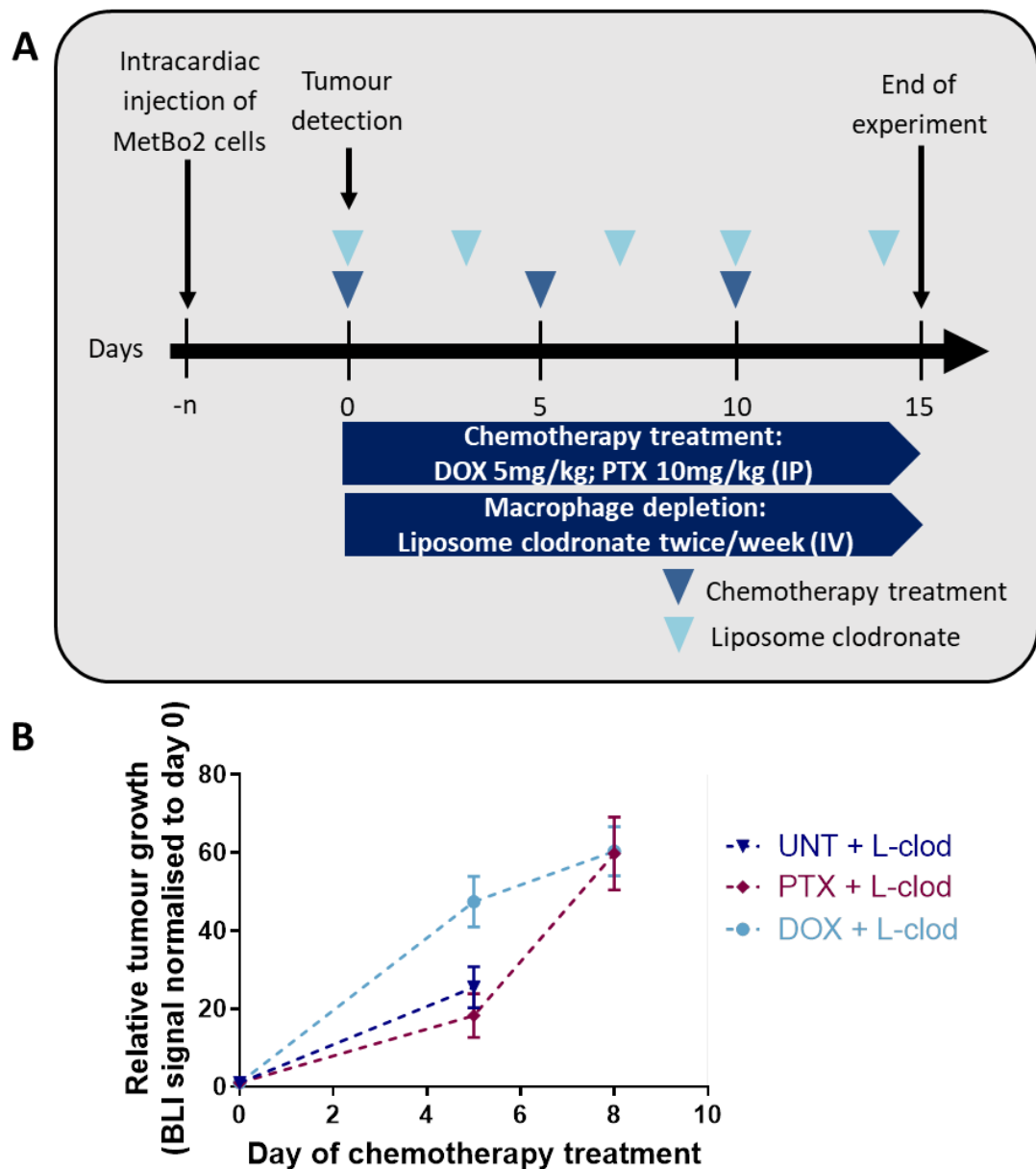


Figure 5.2.9: Macrophage depletion using liposome clodronate does not significantly affect bone metastasis response to chemotherapy during early stages of treatment. A) Experimental design: chemotherapy treatment in MetBo2-derived experimental bone metastasis in FVB mice receiving liposome clodronate (IP, intraperitoneal; IV, intravenous; BLI, bioluminescent imaging). **B)** Quantification of bone metastatic growth throughout chemotherapy treatment with macrophage depletion in comparison to non-depleted controls. Growth was monitored using bioluminescent imaging on day 0, 5 and 8. (L-clod, liposome clodronate). Data shown are mean $\pm$ SEM (n=4-8).

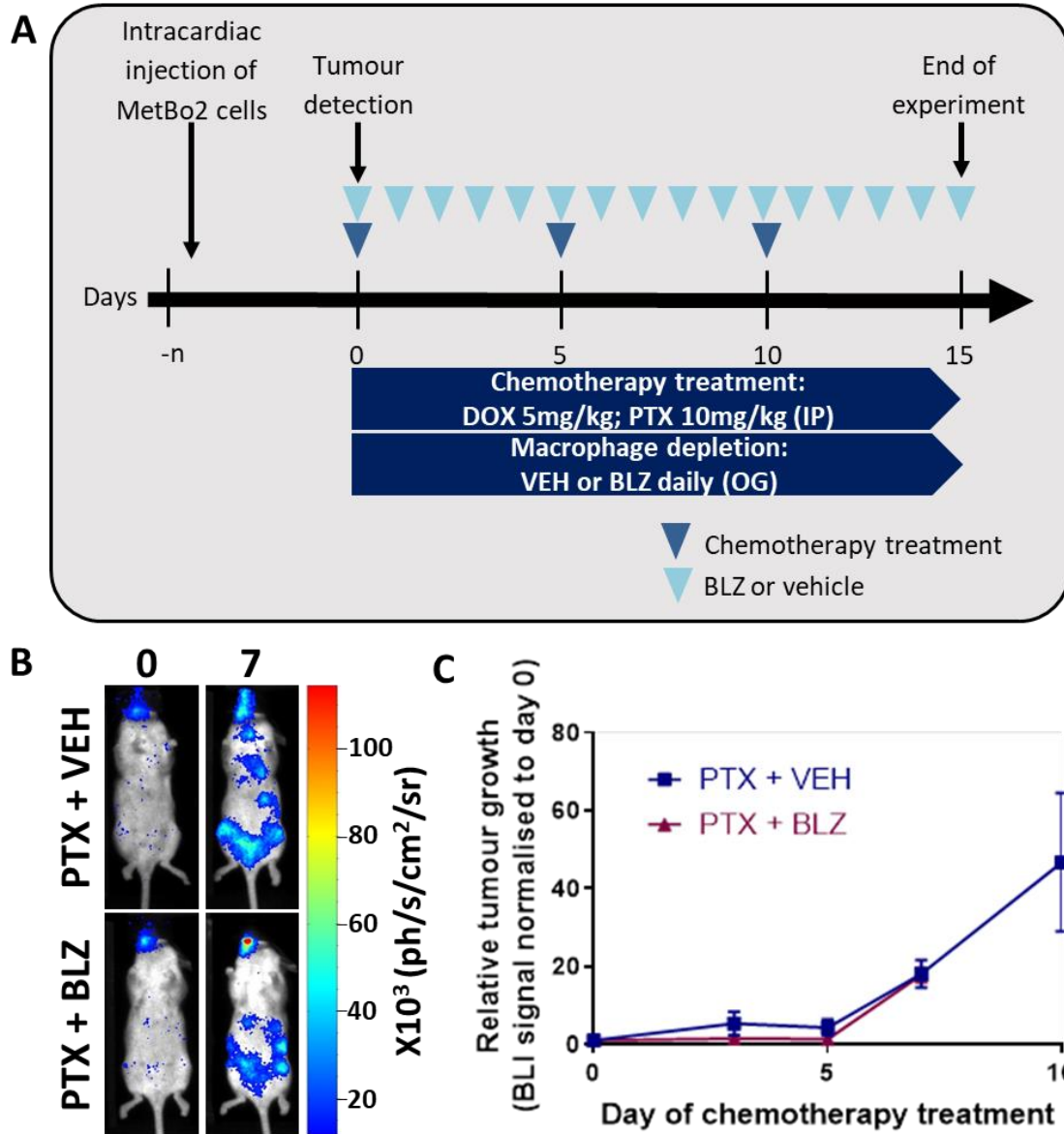


Figure 5.2.10: CSF1R inhibition, BLZ945, does not significantly affect bone metastasis response to paclitaxel. **A)** Experimental design: chemotherapy treatment in MetBo2-derived experimental bone metastasis in macrophage-depleted FVB mice receiving BLZ or vehicle-treated controls (IP, intraperitoneal; BLZ, BLZ945; VEH, vehicle; OG, oral gavage; BLI, bioluminescent imaging). **B)** Representative images of the BLI signal of each group on day 0 and 7 of chemotherapy treatment. **C)** Quantification of bone metastatic growth throughout paclitaxel treatment with and without BLZ treatment. Data shown are mean $\pm$ SEM (n=10-14).

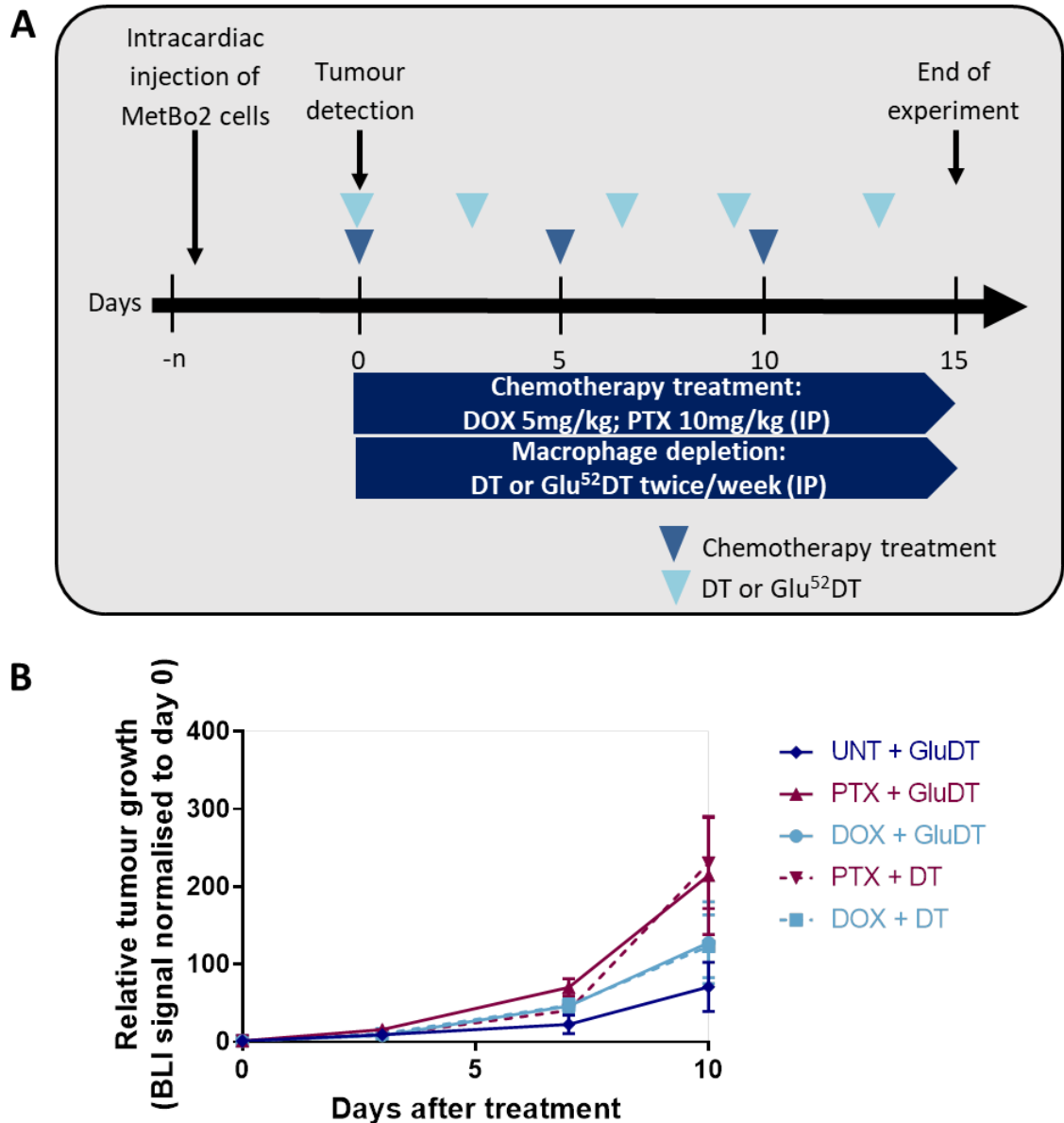


Figure 5.2.11: Depletion of CD169⁺ bone resident macrophages does not affect bone metastasis chemotherapy response. **A)** Experimental design: chemotherapy treatment in MetBo2-derived experimental bone metastasis in CD169-DTR mice. Administration of DT induced CD169⁺ macrophage depletion, whilst control mice received Glu⁵²DT (IP, intraperitoneal; BLI, bioluminescent imaging). **B)** Quantification of bone metastatic growth throughout chemotherapy treatment with and without CD169⁺ macrophage depletion (DT diphtheria toxin). Data shown are mean $\pm$ SEM (n=6).

5.2.2.2 Lymphoid cells

We next investigated whether lymphocytes play a role in the PTX-induced bone metastasis. Previous studies demonstrate that the lymphocyte compartment is altered during the early stages of chemotherapy treatment¹⁴³. Flow cytometry analysis was performed on untreated and PTX-treated bone metastasis as before. Lymphoid cells were stained for CD45, CD3, CD19, B220, NK11, CD8, CD4, CD25 and FOXP3 and used to identify the following cells: B cells (CD45⁺ CD19⁺ CD3⁻ B220⁺), CD4⁺ T cells (CD45⁺ CD19⁻ CD3⁺ CD4⁺), CD8⁺ T cells (CD45⁺ CD19⁻ CD3⁺ CD8⁺), Tregs (CD45⁺ CD19⁻ CD3⁺ CD8⁺ CD25⁺ FOXP3⁺), T-helper cells (CD45⁺ CD19⁻ CD3⁺ CD8⁺ CD25⁻ FOXP3⁻) and NK cells (CD45⁺ CD19⁻ CD3⁻ NK11⁺) (figure 5.2.12). Whilst there were some trends with a decrease in all T cell populations and B cells, we demonstrate that there is no significant difference between the lymphocyte populations in untreated and PTX-treated bone metastasis (figure 5.2.13).

To confirm that there is no functional role of lymphocytes in the chemotherapy response, we performed the chemotherapy-treatment regime in FRag mice (5.2.14a). PTX-treated bone metastasis continued to grow at an increased rate compared untreated metastasis (5.2.14b. figure 5.2.14c). Whilst this was not significantly different, only early stage treatment could be analysed due to adverse effects. In FVB/N mice, the chemotherapy-induced growth was not significant until day 15 with very little difference between day 0 and 10, therefore these results may indicate that lymphocyte depletion further exacerbates the effects of chemotherapy treatment.

Our data demonstrates that the abundance of both the myeloid and lymphoid arm of the immune system is not significantly altered in chemotherapy-treated bone metastasis. This suggests they may not significantly contribute to the chemotherapy response in bone metastasis, and therefore the role of alternative cell types were explored.

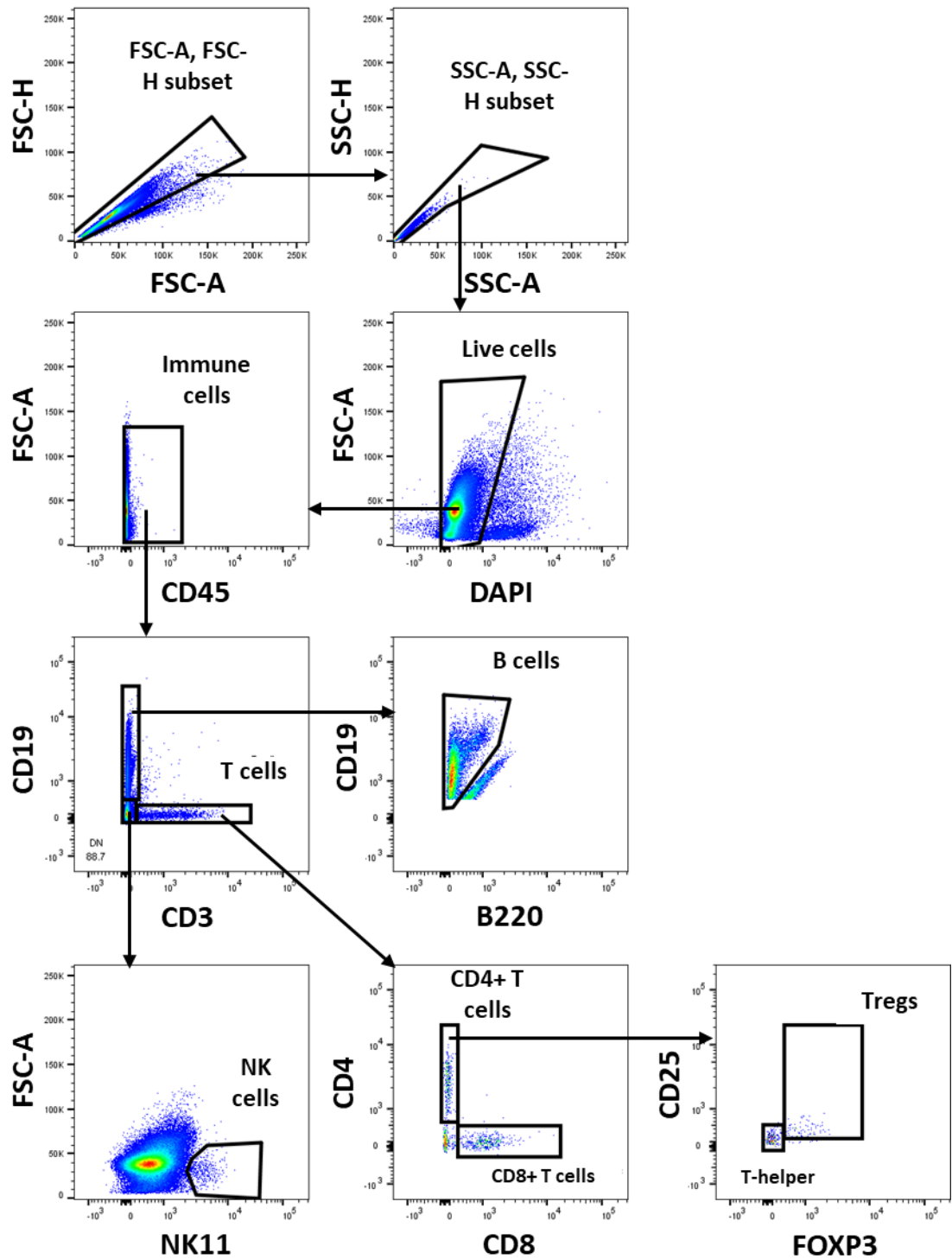


Figure 5.2.12: Representative gating strategy of the lymphoid lineage in bone metastasis.

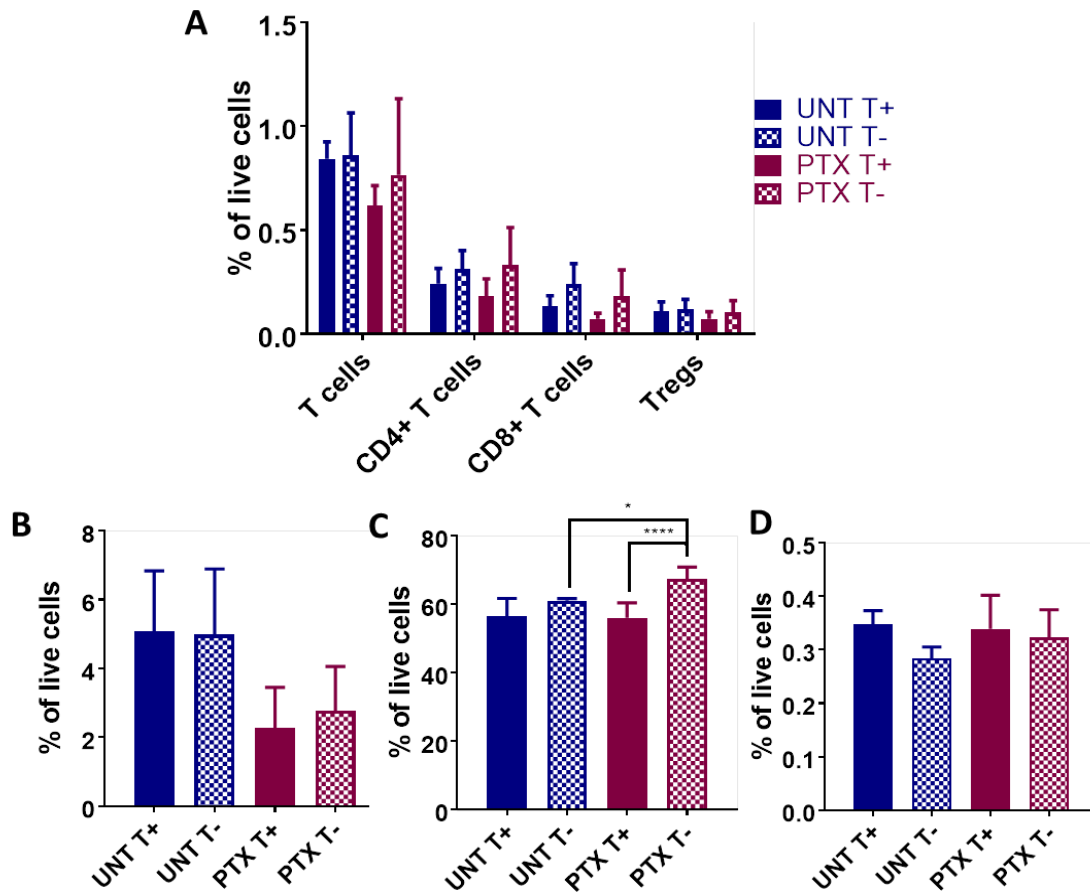


Figure 5.2.13: Lymphocyte populations in bone metastasis are not significantly affected by paclitaxel treatment. Quantification of **A)** T cell, **B)** B cell, **C)** neutrophil and **D)** NK cell populations on day 6 of the chemotherapy treatment regime measured using flow cytometry analysis (T+, tumour enriched region; T-, tumour non-tumour region of bone). Data shown are mean $\pm$ SEM. (n=4).

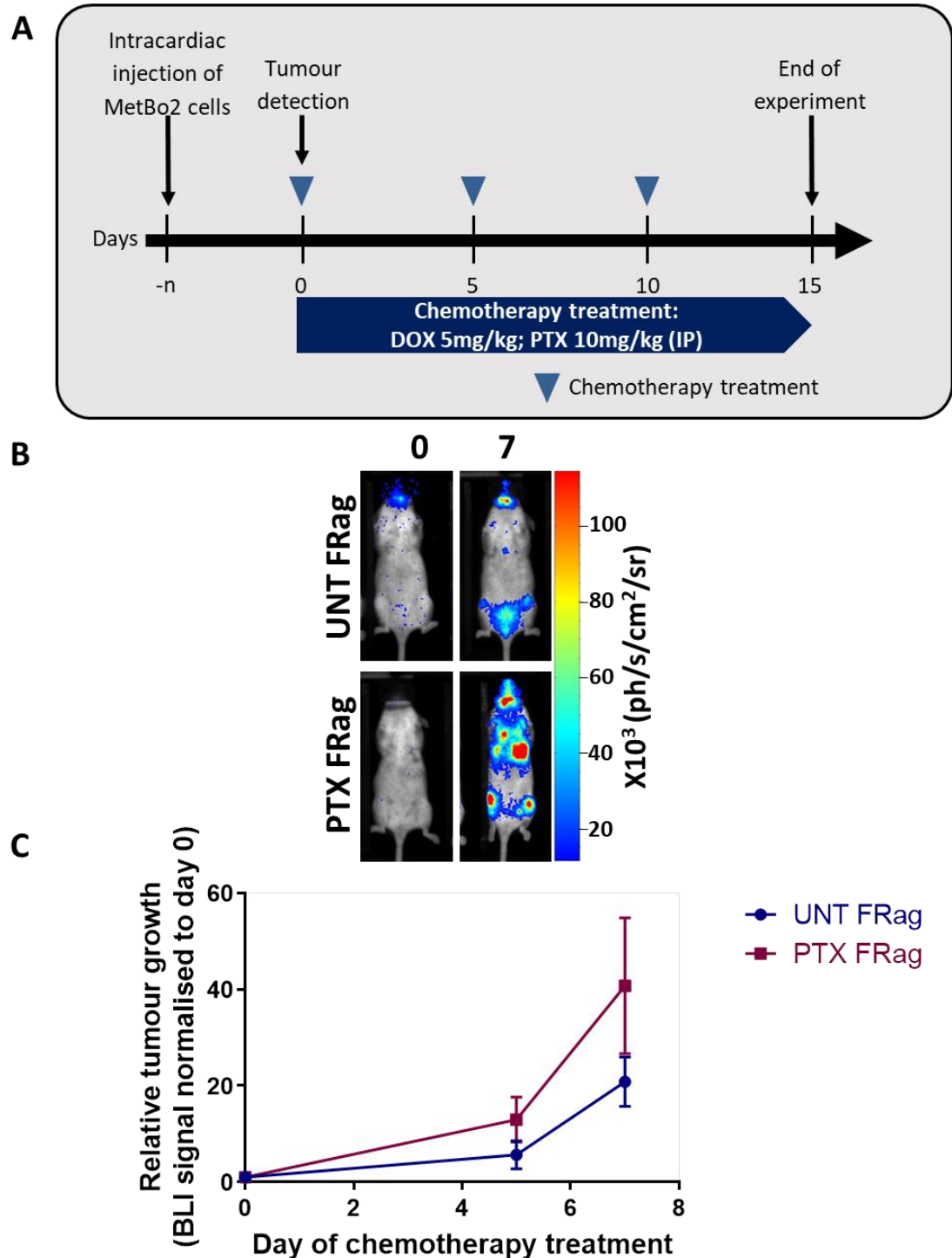


Figure 5.2.14: Paclitaxel-induced bone metastatic growth remains in FReg^{-/-} mice and may be exacerbated. **A)** Experimental design: chemotherapy treatment in MetBo2-derived experimental bone metastasis in FReg^{-/-} mice (IP, intraperitoneal; BLI, bioluminescent imaging). **B)** Representative images of the BLI signal of each group on day 0 and 7 of chemotherapy treatment. **C)** Quantification of bone metastatic growth during untreated and paclitaxel treatment in FReg^{-/-} mice. Data shown are mean $\pm$ SEM (n=8-14).

5.2.3 Role of mesenchymal cells in chemotherapy-treated bone metastasis

Mesenchymal stromal cells are a major component of the TME and have previously been implicated in chemotherapy resistance in several cancer models, including breast and ovarian cancer^{120,121,561}. We also demonstrate in the previous chapter that MSCs, particularly the nestin⁺ subpopulation, display pro-tumorigenic effects on breast cancer bone metastasis. We therefore begin to explore the roles of the mesenchymal lineage in DOX resistance and PTX-induced metastatic growth.

We first characterised the cells of the mesenchymal lineage in untreated and chemotherapy-treatment bone metastasis, and compared the abundance to the TME of MFP tumours. Chemotherapy treatment increases the abundance of CD146⁺ MSCs within bone metastasis, albeit non-significant, whilst this effect does not occur in MFP tumours (figure 5.2.15). Furthermore, the CD146 abundance is significantly higher in PTX- and DOX-treated bone metastasis compared to MFP tumours. FSP⁺ fibroblasts are also significantly increased in chemotherapy-treated bone metastasis compared to MFP tumours. Whilst PTX induces a slight increase in FSP⁺ cells compared to the untreated group, this was not significant (figure 5.2.16). A similar trend was observed in MFP at a much lower number of FSP⁺ cells. This suggests that untreated bone metastasis may exhibit higher abundance of FSP⁺ cells compared to untreated MFP, and that chemotherapy induces the same effect in both. We also analysed the abundance of α SMA⁺ fibroblasts (figure 5.2.17). PTX-treated bone metastasis exhibited a slight, non-significant decrease compared to untreated bone metastasis and there was no significant difference between bone metastasis and MFP tumours with substantial variation between samples in each group. To give further insight into the role of the mesenchymal lineage in the chemotherapy response of bone metastasis, we analysed the correlation between the abundance of each mesenchymal population and BLI or tumour volume, as well as the number of proliferating or apoptotic cells; however, no correlation was observed (data not shown).

To investigate the effect of MSCs and CAFs in chemotherapy response, we in performed *in vitro* proliferation assays (figure 5.2.18a). 1833 cells were seeded at 500cells/well into 48 well plates either alone or in direct co-culture with human MSCs, CAFs or NFs at a 1833:stromal cell ratio of 1:1 to 1:8. After 24 hours to allow the cells to adhere to the plate, the media was replaced with media containing the

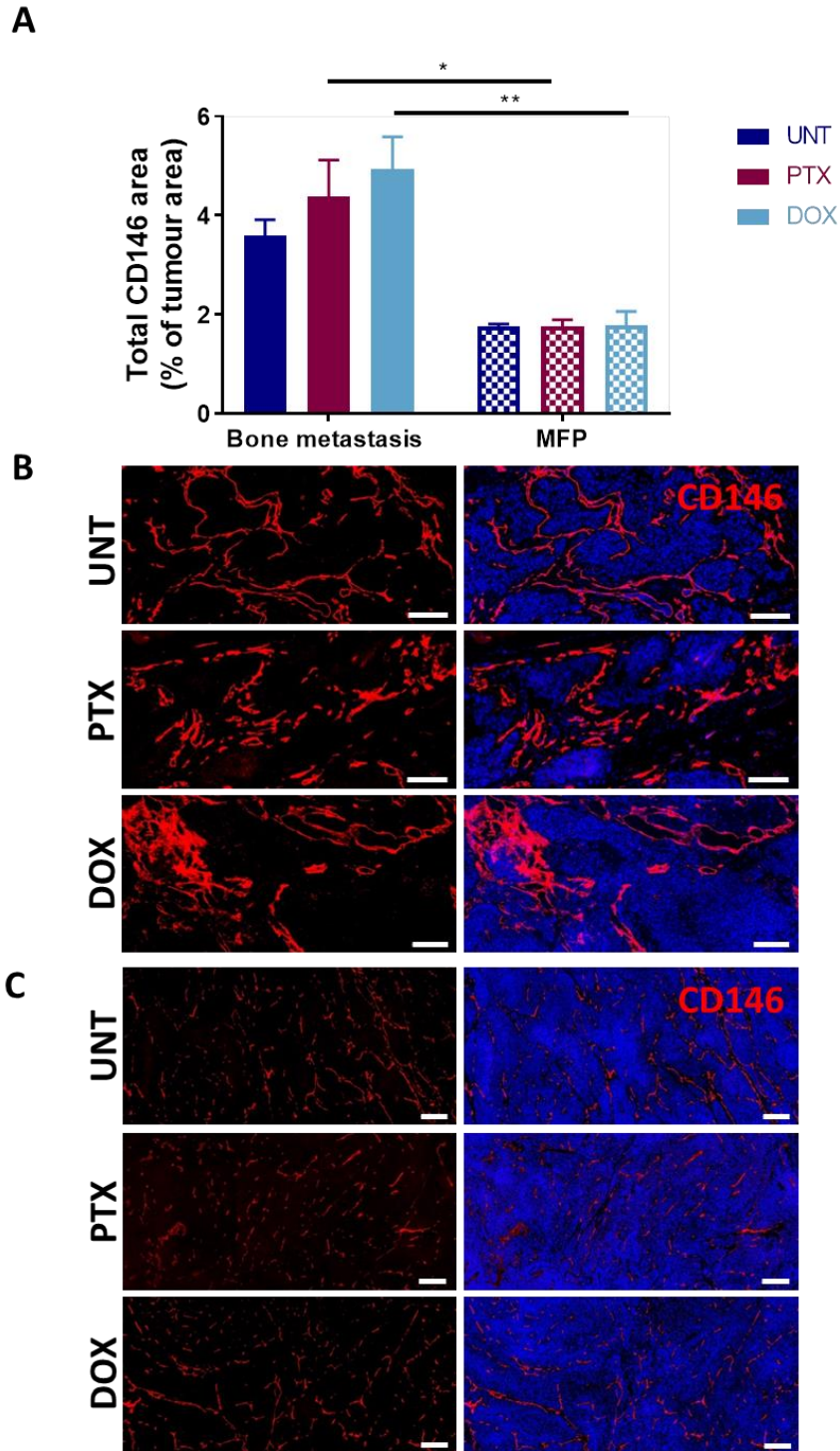


Figure 5.2.15: CD146 abundance is slightly increased by chemotherapy in bone metastasis and is significantly higher than chemotherapy-treated MFP tumours where chemotherapy has no effect on abundance. A) Quantification of CD146 abundance in bone metastasis and MFP tumours on day 15 and day 5 of chemotherapy-treatment regime, respectively. Data shown are mean $\pm$ SEM (* p <0.05 ** p <0.005, two-way ANOVA, bone n =3-8 MFP n =3-8). **B+C)** Representative immunofluorescence images stained for MSC/pericyte marker CD146 (left) and merged with DAPI (right) in untreated, PTX-treated and DOX-treated **B)** bone metastasis and **C)** MFP tumours. Scale bar: 100 μ m.

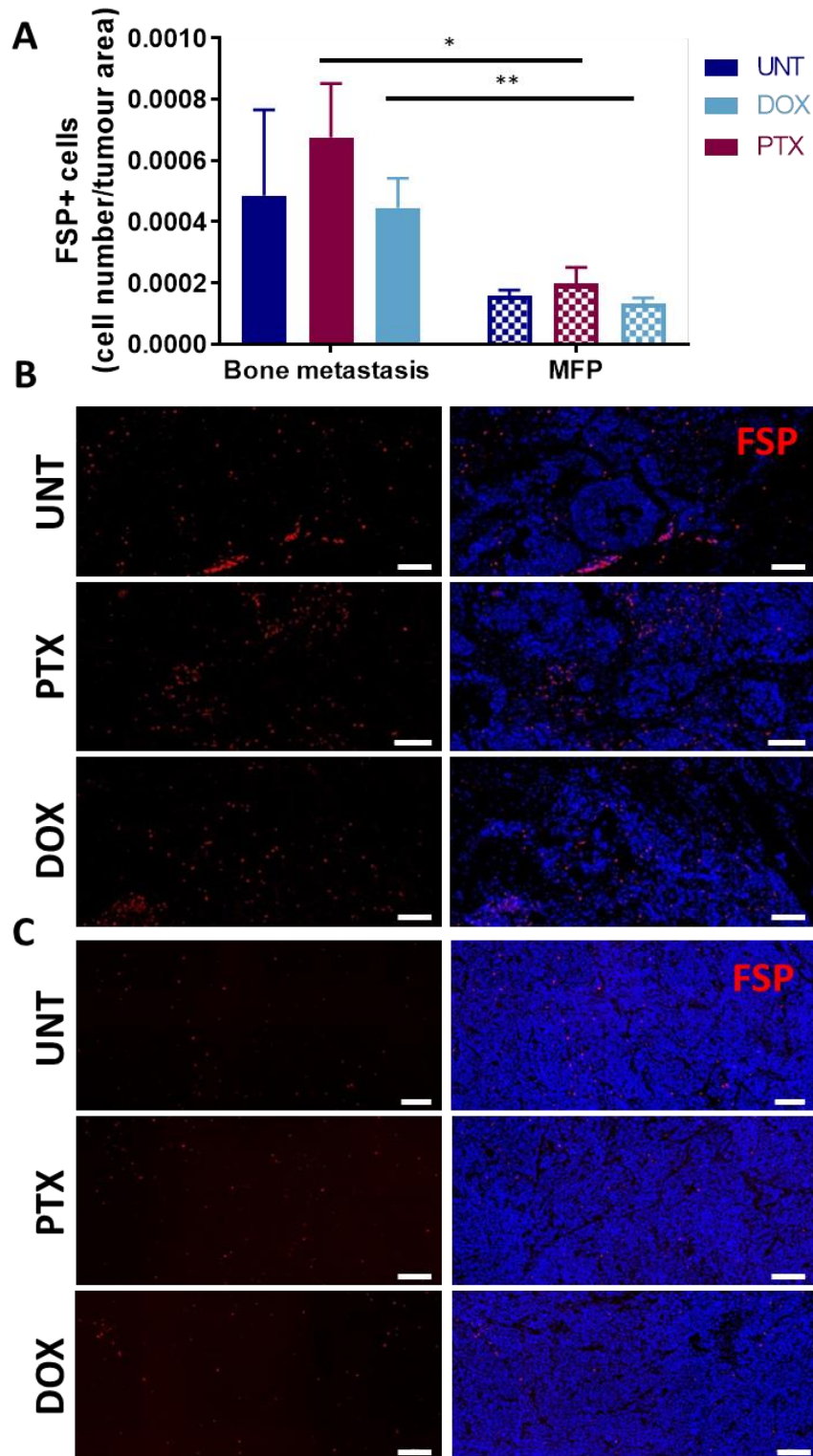


Figure 5.2.16: Bone metastasis contains a greater abundance of FSP+ fibroblasts compared to MFP tumours. **A)** Quantification of FSP+ cells in bone metastasis and MFP tumours on day 15 and day 5 of chemotherapy-treatment regime, respectively. Data shown are mean±SEM (* $p < 0.05$ ** $p < 0.005$, two-way ANOVA bone $n = 3-6$ MFP $n = 3-8$). **B+C)** Representative immunofluorescence images stained for fibroblast marker FSP (left) and merged with DAPI (right) in untreated, PTX-treated and DOX-treated **B)** bone metastasis and **C)** MFP tumours. Scale bar: 100µm.

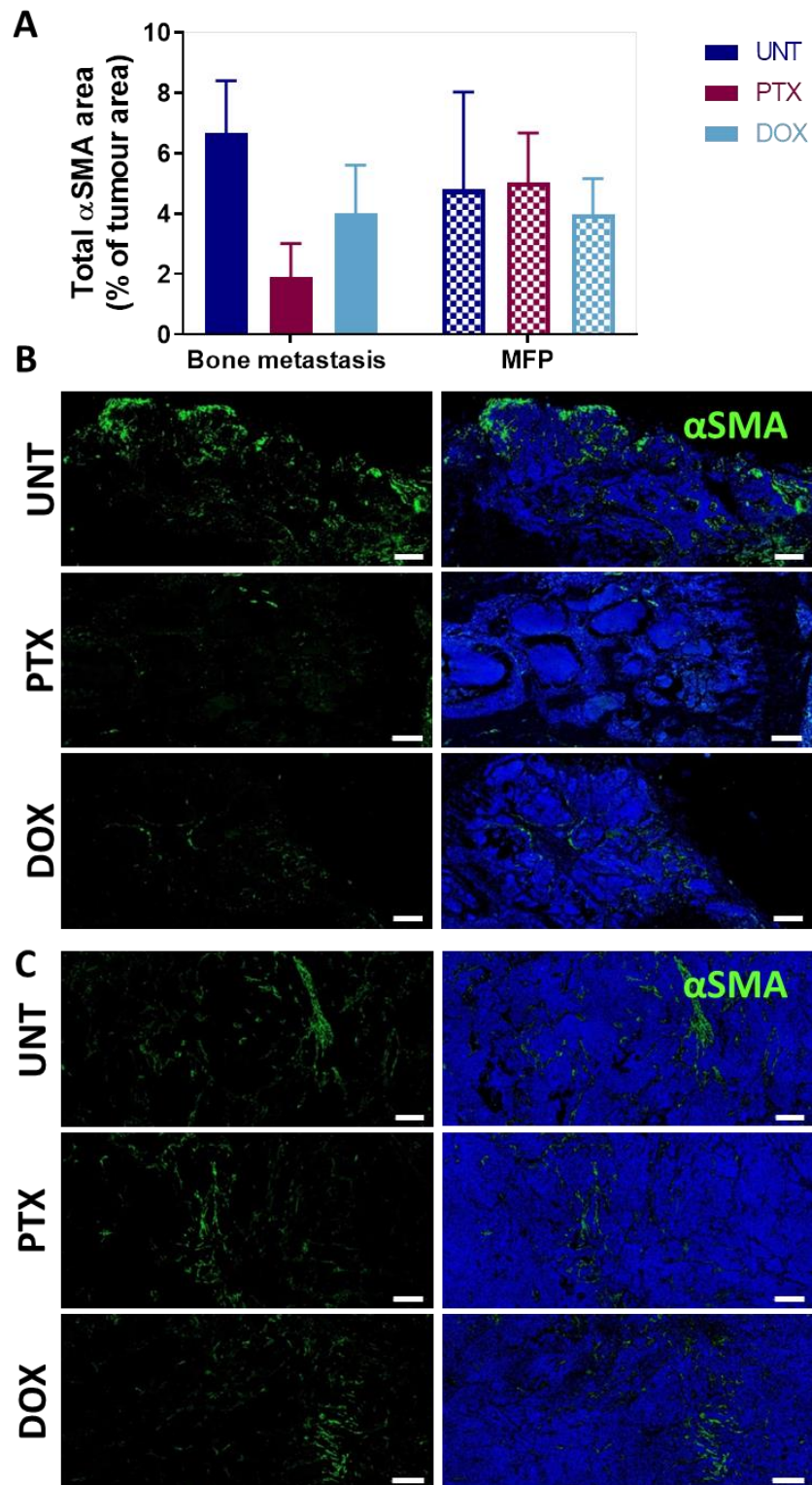


Figure 5.2.17: α SMA abundance is reduced by paclitaxel treatment in bone metastasis. **A)** Quantification of α SMA abundance in bone metastasis and MFP tumours on day 15 and day 5 of chemotherapy-treatment regime, respectively. Data shown are mean $\pm$ SEM (bone n=3-4 MFP n=3). **B)** Representative immunofluorescence images stained for fibroblast marker α SMA (left) and merged with DAPI (right) in untreated, PTX-treated and DOX-treated **B)** bone metastasis. Scale bar: 100 μ m. and **C)** MFP tumours. Scale bar: 200 μ m.

chemotherapy at the appropriate concentration. The chemotherapy concentrations were used as previously described^{552–554}. The cells were cultured for a further 3 days after which a luciferase assay was performed to quantify the number of tumour cells. We demonstrate that both MSCs and fibroblasts induce tumour growth during chemotherapy response with a correlation between the growth and number of mesenchymal cells (figure 5.2.18b-d). MSCs and CAFs induce a significant increase at ratios between 1:2-1:8, whereas the NFs appear to have less effect with a significant increase only being observed at a 1:8 ratio. A transwell assay was used to determine the role of soluble factors from the mesenchymal cells on chemotherapy response (figure 5.2.19a). 1833 cells were seeded in 24 well plates at 1000cells/well and MSCs or fibroblasts were seeded into 0.4µm-pore transwell inserts at the respective ratios. After 24 hours, the media in the wells was replaced with media containing the appropriate concentration of chemotherapy and transwells were transferred to the wells containing the tumour cells. As it has been reported that exosomes that may mediate the effects cannot migrate through the transwell pores, MSC- and fibroblast-derived conditioned media was also used as a variable. The cells were cultured for a further 3 days after which an MTT assay was used to measure the cell viability of tumour cells. The indirect co-culture of the 1833 and mesenchymal cells demonstrates that soluble factors secreted from MSCs, CAFs or NFs alone do not have any significant effect on chemotherapy response (figure 5.2.19b).

We also investigated the effect of chemotherapy treatment on the MSCs and fibroblasts within bone metastasis. We first determined the effect of chemotherapy on MSC and fibroblast proliferation *in vitro*. MSCs or fibroblasts were seeded in 24-well plates at a concentration of 500cell/well. After 24 hours, the media was replaced with media containing the appropriate concentration of chemotherapy. An MTT test was performed after a further 3 days to quantify the number of MSCs and CAFs. We demonstrate that both PTX and DOX reduce the growth of MSCs in a concentration-dependent manner (figure 5.2.20a). In contrast, the number of fibroblasts remained unchanged (figure 5.2.20b). This suggests that MSCs are sensitive to chemotherapy, whilst fibroblasts display resistance. To confirm this in chemotherapy-treated bone metastasis *in vivo*, we quantified the number of proliferating MSCs and fibroblasts by performing co-staining of Ki67 and CD146 or FSP, respectively. Chemotherapy-treated bone metastasis exhibited a reduction in proliferating MSCs in comparison to untreated bone and it is therefore plausible that they may exhibit a protective effect against the

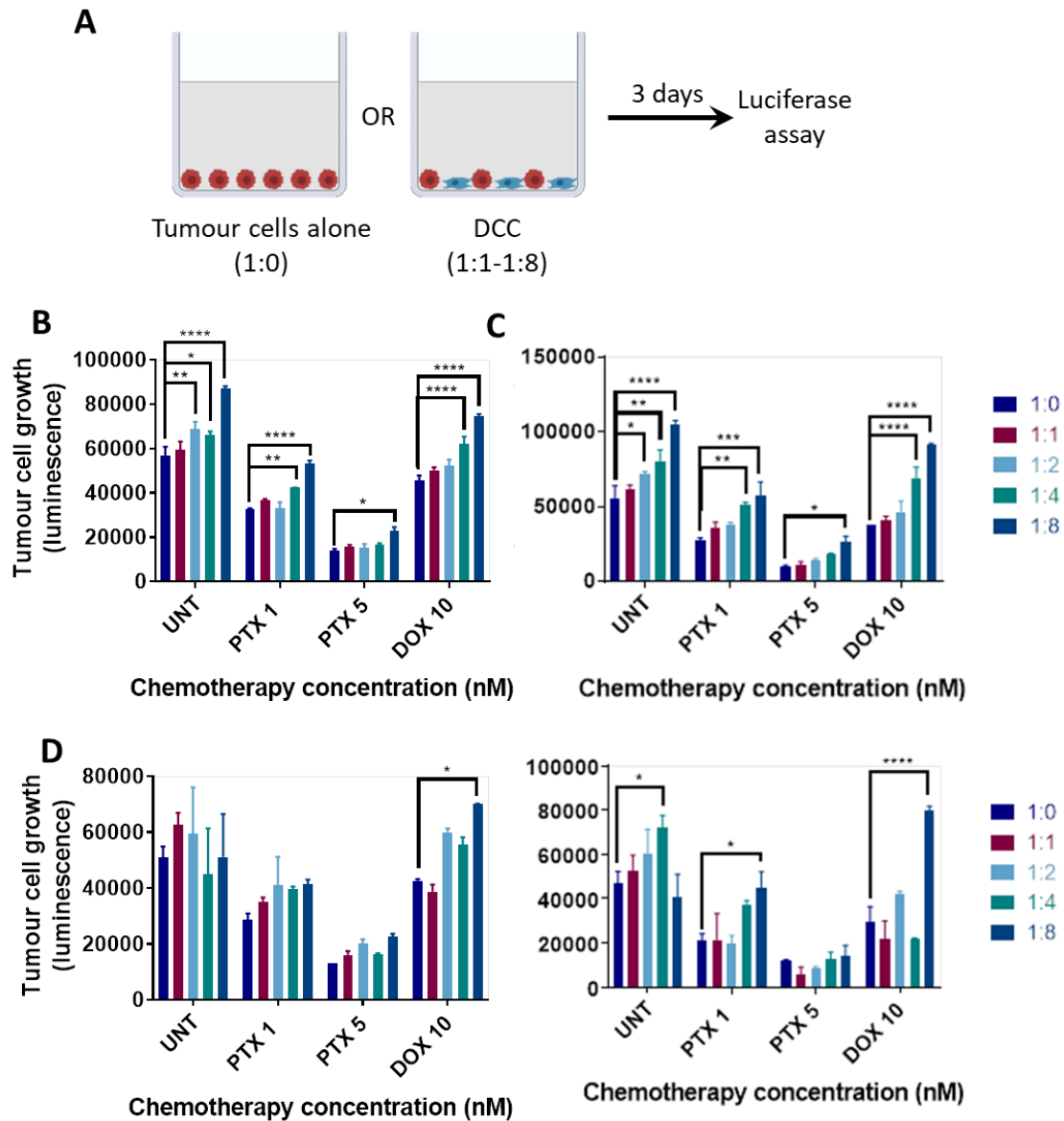


Figure 5.2.18: Direct contact between mesenchymal stromal cells and tumour cells promote chemotherapy resistance *in vitro*. **A)** Experimental design: proliferation assay of tumour cells alone, or in direct-co-culture with MSCs (Red, 1833 cells; blue, MSC or fibroblast. DCC, direct co-culture). **B-D)** Chemotherapy response of 1833 cells in direct co-culture with **B)** HBMSC, **C)** breast cancer-derived CAFs and **D)** fibroblasts derived from normal breast tissue of 2 separate patients at 1833:stromal cell ratio of 1:0-1:8. Cell growth was quantified by luciferase assay on day 3 of chemotherapy treatment. Data shown are mean±SEM (* $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$ **** $p < 0.0001$, two-way ANOVA, $n = 3$).

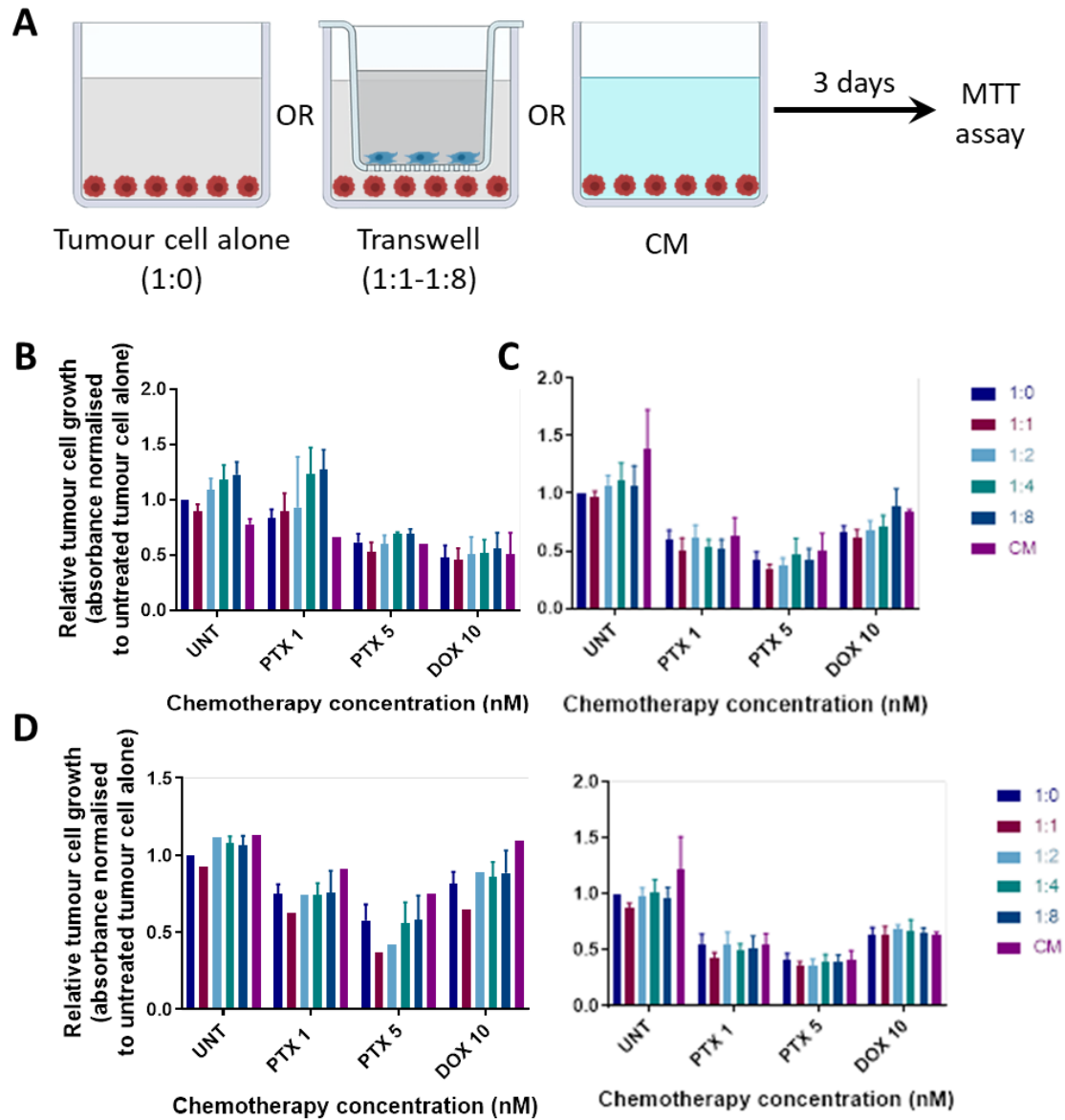


Figure 5.2.19: Secreted factors from mesenchymal stromal cells have no effect on chemotherapy response. **A)** Experimental design: proliferation assay of tumour cells alone, with MSCs in 0.4µm transwells, or MSC-derived CM (Red, 1833 cells; blue, MSC or fibroblast; CM, conditioned media). **B-D)** Chemotherapy response of 1833 cells with transwells containing **B)** HBMSC, **C)** breast cancer-derived CAFs and **D)** fibroblasts derived from normal breast tissue of 2 separate patients at 1833:stromal cell ratio of 1:0-1:8. Cell growth was quantified by MTT assay on day 3 of chemotherapy treatment (CM, conditioned media). Data shown are mean±SEM (* $p<0.05$ ** $p<0.005$ *** $p<0.0005$ **** $p<0.0001$, two-way ANOVA, $n=3-6$).

chemotherapy treatment of MSCs (figure 5.2.21a). In contrast, chemotherapy does not affect the metastasis, and is significant with DOX treatment (figure 5.2.21b, figure 5.2.21b). It should be highlighted that PTX-treated bone metastases do not exhibit a significant decrease in proliferating MSCs, whereas PTX induces a significant reduction in MSC proliferation *in vitro*. The *in vitro* assays did not consider the impact of the cancer cells and other cells of the TME, similar to the effects observed *in vitro*. Interestingly, the number of proliferating fibroblasts was significantly lower in the bone metastasis compared to MFP tumours for each treatment group. Although speculative, these results suggest that proliferation does not seem to be a major source of the increased MSCs in chemotherapy-treated bone metastasis. Similarly, proliferation of FSP⁺ fibroblasts was significantly decreased in bone metastases compared to MFP tumours, whereas the number of FSP⁺ is significantly increased. An alternative source, such as recruitment and differentiation, may therefore occur.

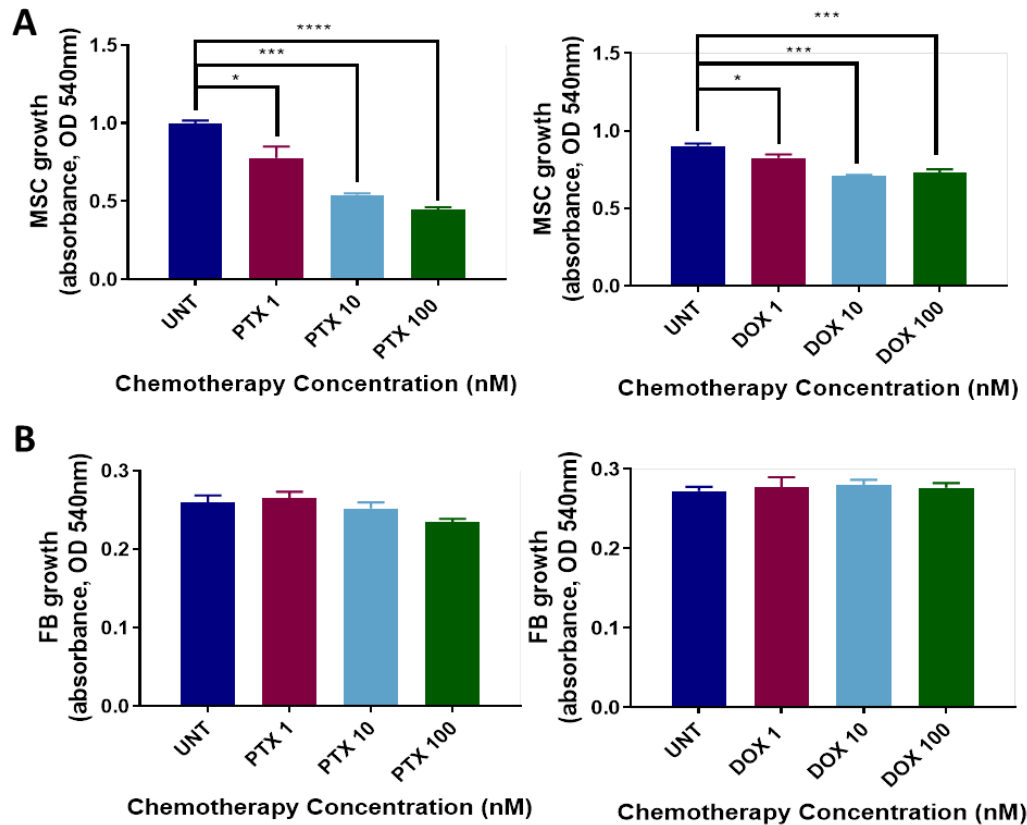


Figure 5.2.20: Chemotherapy reduces MSC proliferation, whilst fibroblasts are resistant *in vitro*. Chemotherapy response of human **A**) MSCs and **B**) fibroblasts *in vitro*. Cell growth was measured using MTT assay on day 5 of chemotherapy treatment. Data shown are mean±SEM (*p<0.05 ***p<0.0005 ****p<0.0001, one-way ANOVA, n=3).

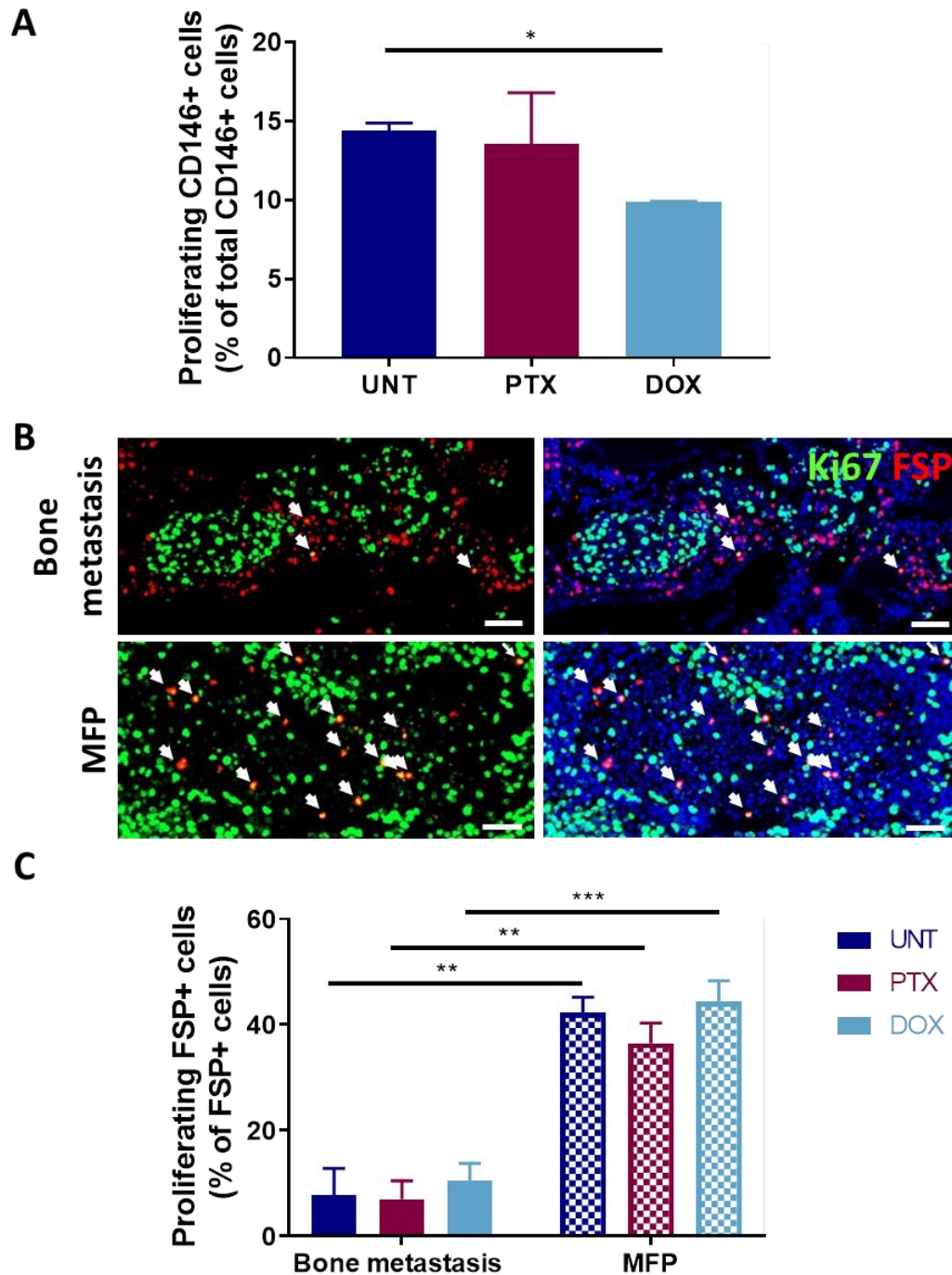


Figure 5.2.21: Chemotherapy reduces MSC proliferation, whilst fibroblasts are resistant *in vivo*. **A)** Effect of chemotherapy treatment on the number of proliferating CD146+ MSCs in bone metastasis calculated by the percentage of CD146+ cells expressing Ki67. Data shown are mean $\pm$ SEM (* p <0.05, one-way ANOVA, n =3-4). **B)** Representative immunofluorescence images stained for FSP and Ki67 (left) and merged with DAPI (right) in bone metastasis (top) and MFP tumours (bottom). White arrows: double positive cells, scale bar: 50 μ m. **C)** Quantification of proliferating FSP+ cells in bone metastasis and MFP tumours on day 15 and day 5 of chemotherapy-treatment regime, respectively. The number of proliferating FSP+ fibroblasts was calculated by the percentage of FSP+ cells expressing Ki67. Data shown are mean $\pm$ SEM (** p <0.005 *** p <0.0005, two-way ANOVA, bone n =3-6 MFP n =3-8).

Previous studies demonstrate that MSCs exhibit a fibroblast-like phenotype during chemotherapy⁵⁶². We therefore also investigated the effect of chemotherapy treatment and chemotherapy-treated tumour cells on MSC differentiation using an *in vitro* differentiation assay (figure 5.2.22a). MSCs were seeded on sterile glass coverslips in 24-well plate at a concentration of 1000cells/well. After 24 hours, the media was replaced with conditioned media derived from chemotherapy-treated tumour cells, which was collected from 1833 cells after 24 hours of chemotherapy treatment. Fresh media with chemotherapy added at the same concentration to that added to the tumour cells was used as control. We demonstrated that both PTX- and DOX- induced α SMA expression in the MSCs, whilst conditioned media from chemotherapy-treated tumour cells prevented this effect (figure 5.2.22b). It is unclear why the conditioned media from chemotherapy-treated tumour cells do not induce α SMA concentration whilst chemotherapy does. It should be considered that the chemotherapy concentration in the conditioned media was reduced as the drug was taken up by the tumour cells. It is also possible that whilst chemotherapy induces α SMA expression, there is a preventative effect of chemotherapy-treated tumour cells and would account for decreased the abundance of α SMA⁺ cells observed in chemotherapy-treated bone metastasis *in vivo*.

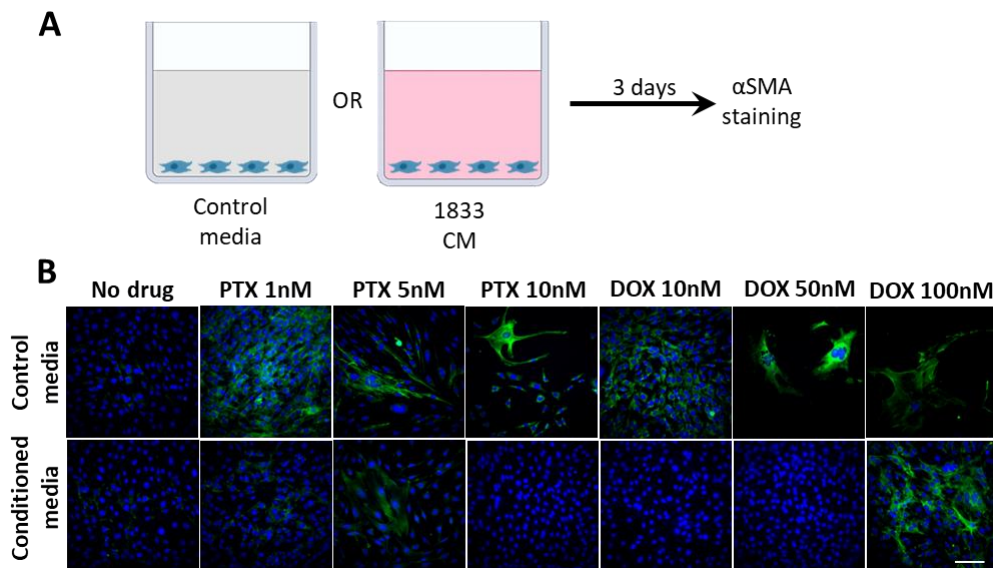


Figure 5.2.22: Chemotherapy alone induces α SMA expression in MSCs, which is attenuated by tumour cell-secreted factors. **A)** Experimental plan: differentiation assay of MSCs in control media or conditioned media derived from untreated or chemotherapy-treated 1833 cells (blue, MSCs; CM, conditioned media). **B)** Immunofluorescence images of MSCs stained for α SMA treated with chemotherapy in control media (top) or conditioned media derived from 1833 cells treated with the same concentration (bottom). Scale bar: 100 μ m. Representative images of 3 separate experiments performed with 3 separate MSC isolations are shown.

Together, these results demonstrate that both MSCs and fibroblasts can promote the growth of bone metastatic cancer cells during chemotherapy treatment. Both populations are increased in bone metastasis compared to the MFP tumours, with a further increase of MSCs induced by chemotherapy treatment in the bone metastasis. This increased abundance may account for the chemotherapy resistance and induced growth observed *in vivo*. We also provide preliminary evidence to indicate that fibroblasts are resistant to chemotherapy, whilst MSCs are sensitive and exhibit reduced growth. Moreover, chemotherapy induces a fibroblast-like phenotype in MSCs that may account for the attenuated proliferation. This illustrates that chemotherapy treatment modulates the mesenchymal lineage in the TME, including proliferation and differentiation, which may subsequently promote resistance or chemotherapy-induced growth, and requires further investigation *in vivo*.

5.3 Discussion

5.3.1 Effect of chemotherapy on bone metastasis

Here we provide evidence that bone metastases are resistant to doxorubicin whilst paclitaxel-treatment promotes chemotherapy-induced growth. We demonstrate that the chemotherapy resistance and chemotherapy-induced growth only occurs in bone metastasis with MetBo2-derived tumours established in the MFP exhibiting chemotherapy-sensitivity, similar to observed in the primary tumours. This indicates the effects in bone metastasis are dependent on the microenvironment in the bone. These differential effects of chemotherapy have been previously observed in prostate cancer bone metastasis where subcutaneous tumour growth was reduced by doxorubicin, whilst no effects were observed in the bone⁵⁶³. However, this is the first time that paclitaxel-induced bone metastatic progression has been observed in established metastasis.

Chemotherapy is given to patients exhibiting breast cancer metastasis to the bone as a palliative treatment where it is thought to restrict the growth of current lesions and reduce the incidence of new lesions developing. It is established that bone metastasis in patients display resistance to chemotherapy. Bone metastasis is rarely eradicated by chemotherapy, whilst some patients exhibit an initial slowing of progression that eventually recovers due to resistance, defined as a partial response. Other patients exhibit no response and the tumour continues to grow. Early studies of breast cancer bone metastasis demonstrate that only 59% patients receiving doxorubicin-based chemotherapy in combination with cyclophosphamide, 5-fluorouracil or vincristine showed response with complete response in 7% and partial response in 52%. This demonstrates that resistance to doxorubicin was observed in 93% of patients either in non-responding or partially responding patients. A more recent study demonstrated that complete remission was only observed in one of 132 patients with breast cancer bone metastasis. All other patients exhibited resistance to chemotherapy with no response and partial remission in 96 and 35 patients, respectively¹.

In contrast, chemotherapy-induced tumour progression has only recently begun to be investigated in other cancer types, particularly in primary cancers where chemotherapy induces changes that promote metastasis (discussed later)⁵⁵⁶. Here, we provide the first preclinical evidence to demonstrate that chemotherapy may enhance growth in tumours already established at the secondary site. Data from the clinic demonstrates

that chemotherapy may reduce the median survival time, but has not been studied directly. In a cohort of patients with bone metastasis, those who had received previous chemotherapy exhibited a median overall survival of 4.0 months from diagnosis of bone metastasis, whilst patients without chemotherapy displayed an overall survival of 6.0 months. Chemotherapy use was documented in 59.9% of the 616 patients included in the study, and the majority (70.9%) exhibited multiple bone metastases. The primary lesions were also mixed and included lung, breast and prostate cancer⁵⁶⁴. Similarly, a study performed in 544 patients with bone metastasis demonstrated that previous chemotherapy reduced the median overall survival to 4.0 months compared to 9.9 months in patients who had not received chemotherapy. Only 9.6% of chemotherapy-treated patients survived 2-years, whilst 31.1% of those who did not received chemotherapy were alive after 2 years⁵⁶⁵. Furthermore, previous chemotherapy exhibits a prognostic hazard ratio of 1.39-1.826^{565,566}. In contrast, a review study of lung cancer patients demonstrated that chemotherapy increased the median overall survival from 7.5 months to 11.4 months. However, this study only analysed data from 118 patients and focussed on lung cancer⁵⁶⁷, whereas previous studies encompassed bone metastasis from a range of tumour origins, including GI, urological, gynaecological, and haematopoietic cancers⁵⁶⁶. Other studies also demonstrate partial response to chemotherapy results in an increased event-free survival, but does not affect overall survival^{1,568}. Notably, they predominantly focussed on doxorubicin, 5-fluoruracil, vincristine and methotrexate treatment, and paclitaxel was rarely included in these studies. Overall, this clinical data supports a tumour-promoting effect of chemotherapy treatment in bone metastasis. It should be highlighted that most of this data is observed in studies investigating the prognostic factors and survival of patient with bone metastasis, and there are no clinical studies that focus on the effects of chemotherapy treatment of bone metastasis. Furthermore, the type of chemotherapy administered to each patient was not documented in many of these studies, and therefore may have exhibited different effects. Most studies also correlated administration of previous chemotherapy treatment of the primary tumour with the effects on bone metastasis, rather than chemotherapy treatment of the bone metastasis, which may contribute to different stages of metastatic development. Whilst primary tumour treatment may have induced changes within the primary tumour or bone to initiate metastasis, treatment of secondary recurrences would exert effects on already-established metastases. Nevertheless, some patients exhibit bone metastasis at the time of diagnosis of the primary tumour and DTCs are detected in the bone marrow of 15-30% of breast cancer

patients at primary tumour diagnosis^{569–571}. Metastatic cells already established in the bone may therefore also be exposed to the effects of primary tumour treatment. It is therefore essential that further preclinical research is carried out in combination with clinical trials to delineate the response of bone metastasis to each chemotherapy type independent of the primary tumour, and to identify the mechanisms that mediate these effects.

In order to determine the how the chemotherapy exerts these growth promoting effects, we next analysed the effects of chemotherapy treatment on the apoptotic and proliferative state of the bone metastatic cells. Apoptosis is the predominant mechanisms of chemotherapy-induced cell death. However, previous studies demonstrate that dysregulation of apoptosis in cancer cells, which is one of the fundamental hallmarks of cancer, reduces the cytotoxicity of chemotherapy resulting in increased cancer cell survival, and thus resistance^{572,573}. In contrast to chemotherapy-treated MFP tumours that exhibit increased apoptotic tumour cells, the number of apoptotic tumour cells in chemotherapy-treated bone metastasis is reduced compared to untreated bone metastasis. This demonstrates that whilst chemotherapy induces apoptosis in the MFP resulting in decreased growth, bone metastasis appear to be resistant to apoptosis. Previous studies have identified a resistance to chemotherapy-induced apoptosis in several human cancers, including breast, colon and brain cancer. This was mediated by expression of anti-apoptotic molecules, such as XIAP, Bcl2 and Bcl_{xL}, as well as upregulation of apoptosis repressor with caspase recruitment domain (ARC), which is encoded by *No/3* and functions to inhibit apoptosis and therefore facilitates chemotherapy resistance^{574–578}. In the PyMT model of breast cancer, endogenous ARC is significantly upregulated in the breast cancer cells. ARC deficiency in PyMT;*no/3*^{-/-} mice results in increased apoptosis during doxorubicin treatment in comparison to PyMT;*no/3*^{+/+} mice, highlighting a role of apoptosis resistance in chemotherapy resistance⁵⁵³.

We also demonstrate that chemotherapy treatment expectedly reduces the number of proliferating tumour cells in the MFP, whilst proliferating cells in bone metastasis are not significantly unchanged. More importantly, bone metastasis exhibit significantly less proliferating cells compared to MFP tumour in each treatment group. It may seem paradoxical that a decrease in proliferating cells may result in chemotherapy resistance or chemotherapy-induced metastasis; however, as chemotherapy targets proliferating cells, the bone metastatic tumour cells may therefore exhibit reduced response. It is

possible that the bone metastasis exhibit a quiescence phenotype mediated by dormancy-inducing niches within the bone^{75,555}, and therefore display a reduced response to chemotherapy. In combination with the resistance to apoptosis, this may contribute to the effects of chemotherapy.

It is important to highlight that the observed differences in apoptosis and proliferation were not statistically significant. In a model of primary breast cancer, decreased proliferation and increased apoptosis was only significantly altered at 24 and 48 hours after paclitaxel treatment, respectively⁵⁵⁷. Analysis of proliferation and apoptosis should therefore be performed at earlier time points throughout the chemotherapy treatment before the difference in tumour size is observed. Alternatively, the changes in apoptosis and proliferation may not play a significant role in the chemotherapy resistance and induced growth. Chemotherapy can also induce cell death via other mechanisms such as necrosis and therefore the inhibition of these alternative cell death mechanisms may contribute the increased bone metastatic growth. This hypothesis is supported by an alternative model of resistant breast cancer where doxorubicin-treated bone metastasis did not affect the abundance of apoptotic or proliferating cells^{579,580}. Other studies also demonstrate that macrophages promote resistance in ovarian and cervical cancer in a mechanism independent of apoptosis and is mediated by other mechanisms, although were not identified⁵⁸¹. Further characterisation of these alternative cell death mechanisms may offer insight into the effects of the chemotherapy on the tumour cells. Immunogenic cell death is an alternative cell death mechanism that tumour cells often undergo after chemotherapy treatment. Apoptotic cells undergo a programmed cell death where the debris is cleared and are therefore unable to elicit an immune response against the antigens associated with the apoptotic cell, defined as immune tolerance. In contrast, cells undergoing immunogenic cell death induce an immune response via the release of damage-associated molecular patterns (DAMPs), such as extracellular ATP, and exhibit endoplasmic reticulum stress, reactive oxygen species production and autophagy^{582,583}. DAMPs are recognised by the immune cells as 'eat me' signals and induce a response to mediate their destruction. Chemotherapy is a potent inducer of immunogenic cell death and it should therefore be considered that the dying cells within the chemotherapy-treated bone metastasis may be releasing soluble factors that are eliciting a response within the immune compartment that is, in fact, pro-tumoral. For example, in a model of melanoma, tumour cells undergoing cisplatin-induced cell death released DAMPs, which subsequently induced upregulation of Tim4 on tumour-associated myeloid cells.

This, in turn, repressed the immune response by activating ingestion, autophagy and removal of the debris from the apoptotic cells from the TME, thus reducing antigen presentation and impairing cytotoxic T-cell mediated response. This prevents destruction of the tumour and therefore permits tumour growth resulting in chemotherapy resistance⁵¹⁴

Chemotherapy, including doxorubicin and paclitaxel, as well as other cancer treatments such as radiotherapy, can also induce a senescence phenotype in cancer cells that survive the treatment, and has been observed in numerous cancer types and models as review by Wang *et al.*⁵⁸⁴. Chemotherapy treated cells often exhibit reduced proliferation, cell cycle arrest in G1 phase, upregulation of cyclin-dependent kinase inhibitors p16 and p21, and a senescence-associated secretory phenotype, which encompasses the enrichment of proinflammatory factors secreted by senescent cells⁵⁸⁴. Together, the arrest of tumour cell proliferation provides a protection from chemotherapy, which targets dividing cells. Recent evidence from the MMTV-Wntl breast cancer model also demonstrates that doxorubicin-induced senescent tumour cells engulfed neighbouring cells, which undergo lysosomal-mediated break down and offers a survival advantage to the senescent cancer cell, including reduced cell death⁵⁸⁵. Moreover, the senescence-associated secretory phenotype factors such as IL6, IL8, CXCL12 and CCL2 that are secreted by senescent cells, including both tumour cells and non-tumoural stromal cells, can further promote tumour progression including proliferation, migration and angiogenesis⁵⁸⁴. This chemotherapy-induced senescence may offer another possible explanation for the persistence of chemotherapy-treated bone metastasis compared to untreated controls where the chemotherapy is selecting for senescent, non-dividing cells that are not only resistance to chemotherapy but also promote survival of other tumour cells through the senescence-associated secretory phenotype. It would therefore be valuable to examine the bone metastasis for senescence including analysis of the cell cycle such as p16 and p21 expression, and characterisation of the senescence-associated secretory phenotype.

Another hypothesis for the effects of the chemotherapy is the selection of a drug resistant clone. Tumours initially exist of a heterogeneous population of genetically diverse subclones that each respond differently to chemotherapy treatment resulting in emergence of a drug resistant population. Previous studies demonstrate that selection of subclones in the bone enhances metastatic growth. In a model of TNBC, Src-

hyperactive tumour cells that thrive on CXCL12 and IGF1 secreted from mesenchymal cells are selected for in the CXCL12-rich bone microenvironment⁹⁵. We show here that the chemotherapy resistance and chemotherapy-induced growth observed in bone metastasis was not due to the survival of a resistant subclone selected from the heterogeneous population of MetBo2 cells as the MetBo2 cells isolated from the chemotherapy-treated bone metastasis regained their sensitivity to chemotherapy *in vitro*. This confirms that the effects of chemotherapy are cell non-autonomous and dependent on the TME, and we therefore sought to identify the compartment of the TME responsible for these effects.

We first investigated the role of the immune compartment during chemotherapy treatment, with a particular focus on the macrophages and lymphocytes. Using immunostaining and flow cytometry we demonstrate that there is no significant change in the abundance of these populations in both short and long term treated bone metastasis. We also used cell depletion protocols to determine the role of the immune populations. Our data demonstrates that depletion of total macrophage population using liposome clodronate or BLZ, or depletion of resident macrophages in CD169-DTR mice, has no significant effect on the response to chemotherapy. Similarly, the chemotherapy-induced growth was still observed in bone metastasis established in FRag mice, demonstrating the lymphocyte deficiency does prevent paclitaxel-induced growth. However, this preliminary data obtained from these depletion assays could only be collected during the early stages of chemotherapy treatment, whilst the chemotherapy-induced effects are observed in the late stage between day 10 and 15. Furthermore, whilst these techniques have been established to deplete macrophages within the lab (Ma *et al.* submitted), the efficiency of the macrophage depletion during chemotherapy treatment within this model should be determined by performing immunostaining and flow cytometry analysis as it is possible the lack of effect is due to poor depletion efficiency. This data is therefore inconclusive and requires further investigation.

The models used to deplete total immune populations resulted in significant adverse effects, including rapid weight loss, resulting in premature termination of the experiments. These adverse effects are most likely due to the combined toxicity of the immunosuppression by the chemotherapy with the additive effect of further macrophage or lymphocyte depletion. Likewise, whilst targeting macrophages using inhibition of the CSF1-CSF1R pathway is an attractive therapeutic strategy, toxicity is a

concern¹⁰⁸. Previous studies utilise a similar macrophage depletion protocol in MFP tumours established from PyMT-derived cancer cells. BLZ was administered daily for up to 8 days and results improved chemotherapy response due to significant the macrophage depletion. However, BLZ was administered prior to chemotherapy treatment and only a single dose of paclitaxel was given⁵⁵⁷. Furthermore, another study that identifies the role of zoledronic acid in enhancing chemotherapy efficacy in breast cancer bone metastasis demonstrates that zoledronic acid is only beneficial if administered after doxorubicin, whereas simultaneous treatment exhibits a less effect⁵⁸⁶. Alterations to the depletion protocol may therefore overcome some of these adverse effects. It may be valuable to initiate the depletion protocols either before the commencement of the chemotherapy, or at a later stage of the chemotherapy treatment to permit depletion between day 10 and 15 of treatment. For the macrophage response, we tested two protocols using liposome clodronate and CSF1R inhibitor, BLZ, to deplete total macrophages, and the CD169-DTR mice to deplete bone resident macrophages only. Alternative depletion models may also be utilised including the use of CD11b-iDTR and CCR2^{-/-} transgenic mice, which would result in depletion of a specific subpopulation of macrophages, leaving other populations intact. Furthermore, the use of the latter would indicate whether recruited monocytes/macrophages serve as origins of macrophages in chemotherapy-treated bone metastasis.

Alternative experimental approaches may also be valuable in characterising the immune compartment. Chemotherapy may induce an activated phenotype in the immune cells, rather than causing recruitment or change in cell abundance. We therefore plan on further characterising the immune populations to identify specific subsets that may be upregulated. Flow cytometry analysis of the markers expressed by populations previously identified in other chemotherapy treated cancer models may identify a specific subpopulation of immune cell involved. RNA-sequencing will also identify specific gene signatures altered in response to chemotherapy. Lineage-specific deletion of the potential molecules, as well as molecular inhibition and knockdown techniques, can then be used to test the function of potential pathways identified. For example, the chemotherapy treatment assays can be performed in transgenic mice with macrophage- or lymphocyte-specific depletion of a molecule. This may identify a specific subpopulation of immune cells activated during chemotherapy treatment and responsible for the chemotherapy resistance or chemotherapy-induced growth.

The mesenchymal compartment may also be important in the chemotherapy response of bone metastasis. MSCs and CAFs, which are increased in bone metastasis compared to the MFP tumours, promote tumour growth and chemotherapy resistance. Using *in vitro* proliferation assays we demonstrate that MSCs and CAFs enhance 1833 growth during chemotherapy treatment in a cell-contact dependent manner, and may account for resistance. PTX-induced growth was not observed as the tumour cells number was not higher than the untreated group. Whilst it should be considered that the co-culture system may not mimic the *in vivo* environment, it may also suggest that another cell type either alone or in combination with MSCs is involved in chemotherapy-induced metastatic growth. Moreover, the MSCs and CAFs displayed a greater effect than NFs with a significant increase observed at lower ratios. This highlights that a cancer-activated phenotype may be important. To confirm the role of MSCs, flow cytometry analysis of the mesenchymal compartment should be performed to provide a detailed analysis of the mesenchymal compartment. It is also important to carry out *in vivo* cell depletion and lineage tracing experiments to confirm the functional role of the mesenchymal cells. Given the role of nestin⁺ MSC population previously observed in bone metastatic growth, it would also be valuable to characterise their abundance in chemotherapy-treated bone metastasis, and determine the effect of nestin⁺ MSC depletion on the chemotherapy response. This was not previously undertaken due to limited numbers of mice available, but may be performed at a later date. It should also be noted that the increase in CD146⁺ MSCs may be along the blood vessels, although the staining is quite diffuse, particularly in the chemotherapy-treated bone metastasis. This may therefore also indicate an increase neoangiogenesis in bone metastasis TME compared to MFP tumours, and therefore may also contribute to chemotherapy resistance or induced growth. Co-staining of CD146 and blood vessel markers such as endomucin or CD31 would give an indication of this. Once the cells responsible are identified, the molecular mechanisms should be investigated using RNA-sequencing and RT-PCR to identify a chemotherapy-induced activated molecular phenotype. The role of potential upregulated molecules can subsequently be confirmed functionally using overexpression and knockdown or inhibition protocols in both *in vitro* proliferation assays and *in vivo* bone metastasis assays.

Finally, an important aspect in understanding the role of mesenchymal cells in chemotherapy resistance and induced growth is to study the effects of chemotherapy exposure on these cells. We therefore analysed the potential effects of chemotherapy on the mesenchymal lineage. We first demonstrate that MSCs are sensitive to

chemotherapy with a dose-dependent decrease in MSCs *in vitro*, and a decrease in the abundance of proliferating MSCs in treated bone metastasis. In contrast fibroblasts are resistant to chemotherapy with no effect of treatment both *in vitro* and *in vivo*. Furthermore, MSCs adopt a fibroblast-like phenotype with the upregulation of α SMA in chemotherapy-treated MSCs. There are a number of studies demonstrating the direct effects of chemotherapy on the mesenchymal lineage with an overall agreement that bone marrow MSCs are sensitive to chemotherapy and exhibit reduced growth during treatment. Bone marrow-derived MSCs treated with paclitaxel exhibit a reduced viability and proliferation compared to untreated controls, although there was no difference between the treatment concentrations. The decreased cell number was associated with an upregulation of growth arrest specific factor 1 (GAS1), which is a key regulator of the cell cycle and restricts division by blocking entry into S phase, and therefore was assumed to contribute to the reduced cell number^{562,587}. In contrast, breast and lung associated fibroblasts were resistant to taxane-based chemotherapy with specific CD10⁺ CPR77⁺ population exhibiting enhanced survival compared to total fibroblasts⁵⁸⁸. MSCs were also sensitive to doxorubicin resulting in a significant reduction of proliferation, although it should be noted that the treated MSCs continued to proliferation, albeit more slowly. This is similar to our observation *in vivo*. Moreover, they also demonstrated that bone marrow-derived MSCs isolated from rats that had received 5mg/kg via intraperitoneal injection exhibit a much smaller, more fibroblast-like phenotype and a decreased ability to undergo osteogenic differentiation in comparison to those isolated from untreated rats⁵⁸⁹. Together with our data, these studies agree that MSCs exhibit reduced numbers after paclitaxel and doxorubicin treatment *in vitro* and appears to be due to inhibition of proliferation and induction of quiescence, although the effects on apoptosis were not observed. Similar anti-proliferative effects resulting in MSC sensitivity has been observed during treatment with other chemotherapeutic agents, including bleomycin, vincristine, etoposide and cytarabine, whilst fibroblasts continue to exhibit resistance^{590,591}. It should be noted that a limited number of reports show that bone marrow MSCs are resistant to busulphan, cyclophosphamide, methotrexate and cisplatin^{590,592}. However, the resistance on bone marrow MSCs appears to be limited to these chemotherapy types.

Bosco et al. demonstrates that paclitaxel-treated MSCs adopt a fibroblast-like phenotype, including a flat, broad appearance, upregulation of fibroblast markers CD9, CD106, integrin α 11, MMP1 and MMP3, and downregulation of MSC markers CD146 and CD166. In contrast paclitaxel treatment reduced the ability to undergo adipogenic

differentiation⁵⁶². Another study demonstrated that chemotherapy-treated MSCs exhibited a diminished ability to differentiate into adipocytes or osteoblasts, although the effects on fibrogenic differentiation were not analysed⁵⁸⁷. The cessation of proliferation and induction of growth arrest coinciding with the differentiation to fibroblast-like cells may account for the reduced cell number where chemotherapy-treated MSCs undergo differentiation as an alternative to proliferation. These fibroblasts subsequently exhibit tumour-promoting effects⁵⁶². Interestingly, we observe a similar fibroblast-like differentiation of MSCs during paclitaxel treatment, observed as an increase in α SMA. Conversely, conditioned media collected from tumour cells treated with the same concentration of chemotherapy does not induce α SMA expression in the MSCs and is supported by a reduction in α SMA⁺ fibroblasts in chemotherapy-treated bone metastasis. However, it is unclear why the conditioned media from chemotherapy-treated tumour cells does not induce α SMA concentration whilst chemotherapy does. It should be considered that the drug concentration in the conditioned media was reduced compared to the control media as the drug was taken up by the tumour cells. Furthermore, the effect of paclitaxel treatment *in vitro*, which is an artificial system, may not necessarily recapitulate the behaviour of the cells *in vivo* as it does not consider the other components of the TME. It is also possible that whilst chemotherapy induces fibroblast differentiation, there is a preventative effect of chemotherapy-treated tumour cells. This would also account for the slight reduction of α SMA fibroblasts in chemotherapy-treated bone metastasis. Moreover, we previously demonstrate the direct contact between tumour cells and MSCs is necessary for α SMA induction in MSCs, which is not considered using the tumour cell-derived conditioned media, and therefore needs to be further investigated. It may also be more valuable to quantify the expression of other fibroblast markers. Adoptive transfer and lineage tracing experiments will also verify the proliferation and recruitment of mesenchymal cells, and differentiation to fibroblasts in bone metastasis *in vivo*. This is an important component of the tumour-stromal interactions that mediate chemotherapy resistance and chemotherapy-induced growth, and it is therefore vital to understand how they are influenced by chemotherapy treatment.

Whilst we were unable to confirm the exact cell responsible for the chemotherapy response observed in bone metastasis, we provide preliminary data to demonstrate that the mesenchymal lineage may contribute. It will therefore be important to perform a detailed characterisation of the TME throughout the chemotherapy treatment. This will identify specific alterations in both the immune and mesenchymal populations as

well as the particular time points where the changes are induced. Previous research in other cancer models may offer some insight into the role of the TME and will therefore be discussed. It is important to note the differential effects between the types of chemotherapy indicating potentially different mechanisms that mediate these effects. We therefore will describe the role of the TME in chemotherapy-resistance and chemotherapy-induced tumour progression separately.

5.3.2 Chemotherapy resistance

Chemotherapy alters the TME by either acting directly on the immune and stromal cells to induce an activated tumour-promoting phenotype, or via the secretion of factors and mediators from tumour cells, defined as the secretome, which acts upon the TME and induces recruitment of cells to the tumour⁵⁹³. Alterations in tumour secretome are observed during chemotherapy treatment, and are essential for cell-cell communication with the TME. Chemotherapy-induced tissue damage also results in systemic release of cytokines and chemokines that stimulates mobilisation of immune and mesenchymal cells to the tumour where they elicit tumour-promoting effects.

5.3.2.1 Immune cells

The role of immune cells in chemotherapy treatment is well studied, and it is now evident that interactions between tumour cells and the immune populations are vital in resistance. TAMs have emerged as potent mediators of chemotherapy response and will therefore be the focus of this discussion. There is now abundant evidence to verify that chemotherapy induces the recruitment of macrophages to the TME in several cancer models where they exert tumour-promoting effects to induce resistance^{143,594}. TAMs infiltrating chemotherapy-treated tumours are often phenotypically distinct from those in naïve, untreated tumours. This includes a preferential polarisation to tumour-promoting M2-like TAMs, which chemotherapy can promote by skewing the differentiation of monocytes. Blockade of macrophage infiltration using CSF1 inhibitors in combination of chemotherapy improves response in many cancers including breast and ovarian^{143,574,595}. For example, the presence of TAMs limit the efficacy of paclitaxel in breast cancer models, whereas chemotherapy response is improved after their depletion^{143,557}. Chemotherapy also induces recruitment of tumour-promoting TAMs to PyMT-derived mammary tumours in a CCR2-dependent manner. CCR2-deficient tumours established in PyMT;CCR2^{-/-} mice exhibit enhanced sensitivity and response during doxorubicin or cisplatin treatment compared to wildtype controls. Although the

exact source of chemoattractant CCL2 was not identified, it was demonstrated that doxorubicin induces expression in a stromal cell population that does not express markers for fibroblasts or endothelial cells⁵⁹⁶. Flow cytometry analysis and immunofluorescence staining demonstrates that docetaxel-treated ovarian tumours also exhibit increased abundance of TAMs compared to untreated tumours. Further characterisation of the TME demonstrates that both CCL2 and CSF1 are upregulated in chemotherapy treated tumours, which are thought to mediate macrophage recruitment, although this was not tested functionally. The combination of docetaxel treatment and macrophage depletion via BLZ945 results in significant reduction in primary tumour growth in a synergistic manner. Metastasis to the lung is also inhibited by macrophage depletion also in a synergistic manner with docetaxel, and may be mediated by a further downregulation of MMP9 and VEGF⁵⁷⁴. This co-treatment was associated with enhanced infiltration of CD8⁺ T cells illustrating the macrophage depletion may enhance the efficiency of chemotherapy by inducing an anti-tumour immune response, but was not tested functionally⁵⁷⁴.

Chemotherapy also induces an activated phenotype in TAMs via the induction of soluble factors from the TME. For example, IL6 and PGE₂ released from chemotherapy-treated tumour cells promote differentiation to an M2-like phenotype that mediates resistance in models of cervical and ovarian cancer. Treatment with platinum-based therapies, carboplatin and cisplatin, induces IL6 and PGE₂ from chemotherapy-treated tumour cells as a result of NFκB activation via the DNA damage response. IL6 and PGE₂ induce upregulation of STAT3 and downregulation of STAT1 in monocytes, respectively, which promotes differentiation to a M2-like macrophage phenotype. This in turn promotes chemotherapy resistance, and could be prevented by COX2-inhibitors or IL6-inhibiting antibodies⁵⁸¹.

It should also be noted that early studies demonstrated that macrophages enhanced sensitivity to chemotherapy. Mantovani *et al.*⁵⁹⁷ demonstrated that the anti-tumour efficacy of doxorubicin is reduced when mice were administered with macrophage toxins, silica and carrageenan. These early reports therefore suggest that macrophages are involved in cytotoxic effects of chemotherapy at least in leukaemia⁵⁹⁷. Similarly, cyclophosphamide also induces release of cytokines, including CCL4, IL8 VEGF and TNF, from chemotherapy-treated leukaemia cells, which exhibit an acute secretory activating phenotype. These cytokines act as chemoattractants to recruit anti-tumour macrophages that exhibit phagocytic activity in the bone, thus enhancing

response⁵⁹⁸. However, the observed anti-tumour effects of TAMs were context-dependent and restricted to limited tumour and chemotherapy types.

The overall consensus is that TAMs recruited or activated during chemotherapy significantly contribute to resistance through several mechanisms. During treatment, TAMs cooperate with other cells, particularly T cell populations, to create an immunosuppressive environment^{143,595}. As chemotherapy-induced cell death is partially dependent on the presentation of tumour antigens to the T cells, which elicits an anti-tumour response, inhibition of the cytotoxic T cells by TAMs induces resistance⁵⁹⁴. Many cancers that display poor response to chemotherapy, including breast cancer, have a high TAM abundance and low infiltrating cytotoxic T cells, further supporting their role in resistance¹⁴³. Moreover, an immunosuppressive effect is induced by the upregulation of immune checkpoints and is often mediated by TAMs. Alternatively, TAM-derived cytokines and enzymes induced during chemotherapy treatment can promote tumour growth and survival by acting directly promoting tumour proliferation, by inhibiting the DNA damage response or inducing angiogenesis^{599,600}.

5.3.2.1.1 *Suppression of the cytotoxic T cell response*

The suppression of the T cell response by TAMs during chemotherapy response was first highlighted by DeNardo *et al.*¹⁴³ in a model of breast cancer using a combination of human samples and preclinical data. An inverse correlation between the CD68⁺ macrophages and CD8⁺ cytotoxic T cells was observed in chemotherapy treatment breast cancer samples, and an immune profile characterised by CD68^{high}CD4^{high}CD8^{low} was indicative of a poor chemotherapy response. This led them to hypothesize that TAMs may inhibit the infiltration of cytotoxic T cells during chemotherapy leading to an impaired response. Using the MMTV-PyMT model of breast cancer, they demonstrate that paclitaxel treatment induced CSF1 and IL34 secretion from the mammary epithelial cells and subsequent recruitment of TAMs to the tumour, resulting in resistance. Blockade of the TAM recruitment using CSF1-CSF1R inhibitors enhanced the chemotherapy response and was dependent on the release of T cell immunosuppression observed as increased infiltration of cytotoxic CD8⁺ T cells in the TAM-depleted tumours¹⁴³.

Doxorubicin-treated lung cancers also exhibited increased TAM infiltration that promoted chemotherapy resistance via suppression of the CD8⁺ T cell response in an IL34-dependent manner. IL34 was upregulated in human lung cancer samples treated

with cisplatin or doxorubicin, and correlates with poor prognosis. Preclinical models demonstrate that IL34 secretion from tumour cells in response to chemotherapy induces an immunosuppressive phenotype in TAMs and subsequently resistance. Treatment of monocytes with conditioned media derived from doxorubicin-treated tumour cells promotes differentiation into a M2-like phenotype, which exhibits increased ability to suppress of the CD8⁺ T cell response. Inhibition of IL34, or its receptor CSF1R, prevented these effects, and tumours established from IL34-deficient cells did not exhibit TAM infiltration during chemotherapy treatment. Moreover, IL34 also acts directly on tumour cells to promote survival via the CSF1R-mediated AKT signalling⁶⁰¹. Together these studies confirm a functional role of tumour-derived IL34 in TAM-mediated immunosuppression during chemotherapy resistance.

Similarly in PyMT mammary carcinomas, co-treatment of chemotherapy with macrophage inhibitors also prevents resistance to paclitaxel and carboplatin by slowing primary tumour growth and reducing pulmonary metastasis⁵⁹⁵. These effects were also dependent on the increase of CD8⁺ T cells in macrophage-ablated tumours. The monocyte-derived TAMs recruited to the tumours are a major source of IL10 that represses IL12 release from dendritic cells, which would otherwise stimulate a T cell-mediated response against DAMPs released from apoptotic cancer cells. TAM-mediated IL12 suppression therefore subsequently reduces T cell response against the tumour cells resulting in resistance. Comparably, patient samples displaying low IL12 levels do not respond well to chemotherapy, whereas high IL12 expression correlates with complete response in breast cancer patients⁵⁹⁵. In contrast, in an alternative subcutaneous fibrosarcoma model, response to doxorubicin was independent of dendritic cell-secreted IL12. Instead response was dependent on signalling pathways involving IFN γ , IFN β and IL17, which mediates generation of CD8⁺ T cells, dendritic cells and $\gamma\delta$ T cells, respectively⁵⁹⁴. This demonstrates that these effects were differential between tumour and chemotherapy type, and highlights the need to investigate each independently.

The complement pathway is also a critical mediator of the TAM phenotype and immunosuppression during the chemotherapy response. In a model of squamous cell carcinoma, urokinase⁺ population of TAMs regulated the release of C5a, which acts on the C5aR1 on macrophages and mast cells and subsequently suppress CD8⁺ T cell cytotoxicity. Whilst treatment as a single agent was ineffective, therapeutic inhibition of the C5aR1 receptor during paclitaxel treatment reduced tumour growth and enhances

sensitivity. This was associated with macrophage repolarisation to an anti-tumour M1-like phenotype and IFN γ release, as well as an increased CD8 $^{+}$ T cell infiltration to mediate an ant-tumour response⁶⁰².

TAMs also modulate chemotherapy resistance in pancreatic cancer via the suppression of a T-cell mediated response. Gemcitabine induces macrophage infiltration in pancreatic tumours due to chemotherapy-induced expression of CSF1 and CCL2 in the TME. Targeting of TAMs and inflammatory monocytes via the inhibition of CSF1R or CCR2, respectively, improves efficiency of chemotherapy and inhibits metastasis. This is in a synergistic manner where macrophage inhibition has little effect on untreated tumour growth, but significantly reduces growth in combination with chemotherapy treatment, suggesting that chemotherapy must first induce an infiltration. Chemotherapy-induced TAMs acted to suppress T cell function as TAMs isolated from tumours suppressed proliferation and function of CD8 $^{+}$ T cells. Notably, both macrophages from untreated tumours and tumour-naïve bone marrow-derived macrophages had less effect highlighting the importance of an activated phenotype induced by the chemotherapy in the tumour. Macrophage-depletion reversed the TAM-mediated immunosuppression and resulted in increased recruitment of CD4 $^{+}$ and CD8 $^{+}$ T cells and reduced FOXP3 $^{+}$ Treg cells in chemotherapy treated tumours compared to non-depleted controls. Furthermore, the use of CD8 $^{+}$ -depleting antibodies in combination with CSF1R inhibition and gemcitabine treatment prevented the therapeutic effect of macrophage depletion. Together, this not only demonstrates that TAMs recruited to gemcitabine-treated tumours suppress CD8 $^{+}$ T cells, but also that the therapeutic efficiency of macrophage depletion was dependent on CD8 $^{+}$ T cell recruitment to macrophage depleted tumours⁵⁹⁹.

The inverse relationship also exists where infiltrating lymphocytes in the immunosuppressive TME also regulate the tumour-promoting properties of macrophages in breast cancer. CD4 $^{+}$ T cell-derived Th2 cytokines, particularly IL4, promotes the M2-like phenotype. TAMs isolated from tumours established in CD4-deficient or Rag1-deficient;PyMT mice exhibited elevated expression of M1 cytokines in comparison to controls⁵¹⁹. It was recently demonstrated that B cells also regulate the phenotype of TAMs in squamous cell carcinoma. Although B cell depletion using anti-CD20 monoclonal antibody was ineffective as a single therapy, the anti-tumour response was improved when giving in combination with both platinum- and taxol-based chemotherapies. This was dependent on repolarising of TAMs from a M2-like to

M1-type phenotype, which subsequently secreted angiostatic cytokines CXCL10 and CXCL11. The M1 macrophages also induced the infiltration of CD8⁺ T cells, which were recruited to the TME in a CCR5-dependent manner, and exerted an immune response against the tumour cells. Together the anti-angiogenic response and repression of the immunosuppressive environment sensitised established tumours to cytotoxic agents⁶⁰³. Furthermore, B cells also released extracellular vesicles in response to chemotherapy that attenuated CD8⁺ T cell responses leading to systemic immunosuppression and thus chemotherapy resistance in both patients and mice. ATP released from dying tumour cells after chemotherapy treatment, including oxaliplatin, doxorubicin and cyclophosphamide, is hydrolysed by the extracellular vesicles and converted into adenosine, which in turn impairs CD8⁺ T cell function. As these extracellular vesicles were released in response to HIF1 α , targeting HIF1 α promoted chemotherapy response⁶⁰⁴.

In contrast to the above studies, Webb *et al.* also demonstrated that macrophages mediated chemotherapy resistance in a T cell-independent manner. Firstly, macrophages were recruited to neuroblastoma xenografts treated with the chemotherapy, topotecan, via chemotherapy-enhanced release of CSF1 from neuroblastoma cells. Here the macrophages promoted chemotherapy resistance by supporting tumour growth, which could be inhibited by macrophage blockage. Similar tumours established in T cell-deficient NOD/SCID mice still exhibited inhibition of chemotherapy resistance after macrophage depletion. In these mice, combined treatment with CSF1R inhibitor BLZ945 and two CSF1R neutralising antibodies still prevented chemotherapy resistance and improved anti-tumour response in a synergistic manner. This demonstrates that macrophage depletion can improve chemotherapy response without requiring T lymphocytes⁶⁰⁵.

Macrophages also cooperate with neutrophils in chemotherapy resistance. Depletion of TAMs in combination with neutrophils further sensitises mammary tumours to platinum-based chemotherapies resulting in elimination of the cancer. Salvagno *et al.*⁶⁰⁶ utilised a monoclonal antibody to specifically inhibit CSF1R in combination with anti-Ly6G in a murine breast cancer model. Whilst the inhibitors do not affect growth or metastasis of untreated tumours, they improve the response and anti-tumour efficacy to cisplatin chemotherapy. CSF1R inhibition also stimulates the induction of the intratumoral inflammatory type I interferon response. Platinum-based chemotherapies including cisplatin and oxaliplatin both synergised with CSF1R targeting, where docetaxel did

not, despite the induction of IFN α in the docetaxel treated tumours. Furthermore, whilst CSFR1 inhibition alone does not engage an endogenous anti-tumour T cell response, the combination of both macrophage and neutrophil inhibition increases the number of CD8⁺ T cells. Together, the combined induction of the TME into a type I IFN-enriched environment during CSF1R inhibition and relief of the neutrophil-dependent T cell-mediated immunosuppression leads to an improved therapeutic efficacy of cisplatin⁶⁰⁶.

MDSCs are also implicated in chemotherapy resistance by creating an immunosuppressive environment. Through the use of *in vitro* assays and *in vivo* experiments, Rong *et al.*⁶⁰⁷ demonstrated that breast cancer cells secrete PGE₂ in response to chemotherapy. The tumour-derived PGE₂ stimulates recruitment and expansion of MDSCs by acting on the EP2/EP4 receptors, which subsequently inhibits the CD4⁺ T cell response characterised by reduced proliferation and IFN γ release. PGE₂-EP2/EP4 interactions also stimulates the production of miR10a in a protein kinase A-dependent manner, which promotes further expansion and activation of MDSCs, suggestive of a positive feedback loop to maintain chemotherapy-resistance. Interestingly, doxorubicin-resistant breast cancer cells secrete significantly higher levels of PGE₂ than sensitive cells, whereas untreated tumour cells do not exhibit these effects⁶⁰⁷. A similar effect was also observed in a pancreatic cancer model where chemotherapeutic agents, including gemcitabine and 5-fluorouracil, induced enrichment of MDSCs in the tumour via the secretion of GM-CSF, and suppressed T cell response to enable tumour growth. Inhibition of GM-CSF using neutralising antibodies prevented these MDSC-mediated effects, restored T cell proliferation and therefore augmented the efficacy of chemotherapy treatment⁶⁰⁸. Other studies also demonstrate that doxorubicin decreases the MDSC abundance, whilst the NK and cytotoxic T cells were increased. This was due to cytotoxic effects on MDSCs, which impairs the MDSC-mediated suppression of the T cell response, and thus enhances the anti-tumour immune response⁶⁰⁹. In a model of 4T1-derived breast cancer, MDSCs and M1-like macrophages were expanded in tumours that responded to docetaxel in comparison to poor responders. They also exhibited decreased M2-like immunosuppressive TAMs and increased cytotoxic CD8⁺ T cells. Furthermore, *in vitro* assays demonstrate that docetaxel-treated MDSCs exhibit an M1-phenotype and could enhance cytotoxic T cell responses. This suggests that MDSCs are a component of the anti-tumour effects of docetaxel⁶¹⁰ and therefore their role remains disputed.

Chemotherapy may also mediate the T cell population, particularly Tregs, independent of the TAM and MDSC response. In a model of colorectal cancer, 5-fluorouracil treatment resulted in recruitment of immunosuppressive Tregs to the TME via the secretion of chemotherapy-induced CCL20 from the tumour cells. CCL20 upregulation was dependent on activation of FOXO1/CEBPB/NF- κ B signalling in the cancer cells, which are all associated with chemotherapy resistance and poor survival of colorectal cancer patients. Co-treatment of CCL20 inhibitor with 5-fluorouracil prevented tumour progression and restored chemotherapy sensitivity in a murine model, suggestive of a potential therapeutic target⁶¹¹. Bleomycin also stimulates the production of TGF β 1 that induces the expansion of immunosuppressive CD4⁺ CD25⁺ FOXP3⁺ Treg cells that permits further growth. These effects were reversed by inhibiting TGF β with neutralising antibodies resulting in sensitisation⁶¹².

Lastly, recent evidence demonstrates that alterations in the NK cells that contribute to immunosuppression are involved in chemotherapy resistance. Transcriptome analysis of high-grade serous ovarian carcinoma samples before and after treatment with neoadjuvant platinum and taxane-based chemotherapies demonstrated that untreated and treated samples cluster separately and are immunologically distinct. This was particularly significant in the NK cell population, which exhibited an activated phenotype. This is supported by a preclinical model of ovarian cancer where the NK activation was enriched after cisplatin treatment⁶¹³. This highlights that whilst we did not observe a change in the abundance of the immune populations chemotherapy-treated bone metastasis, a similar activated phenotype may occur in our model.

5.3.2.1.2 Upregulation of immune checkpoints

The upregulation of immune checkpoint molecules PD1, PDL1 and CTLA4 after chemotherapy treatment also mediates an immunosuppressive environment and augments immune tolerance, and has been reported in several cancers. For example, analysis of breast cancer patient samples demonstrated that PDL1 expression was observed in 60% of patients receiving neoadjuvant chemotherapy, whereas it was expressed in only 37% patients who did not receive chemotherapy prior to surgical removal^{614,615}. Preclinical experiments demonstrate that paclitaxel as well as etoposide and 5-fluorouracil increases surface expression of PDL1 in breast cancer cells in a dose-dependent manner and promoted PD1⁺ CD8⁺ T cell apoptosis. Co-culture of T cells with chemotherapy-treated breast cancer cells induced T cell apoptosis, which was not observed with untreated cancer cells. Inhibition of PDL1 also prevented these effects.

Whilst they did not perform *in vivo* studies to confirm these findings, it indicates that chemotherapy may create an immunosuppressive environment to permit resistance by inducing PDL1-PD1-mediated T cell apoptosis. Anti-PDL1 or PD1 treatment may serve as an adjuvant therapy to prevent resistance⁶¹⁶.

A similar study also illustrates that neoadjuvant chemotherapy treatment of epithelial ovarian cancer not only induces an increase of tumour infiltrating lymphocytes, but also induces PDL1 expression in immune infiltrate and tumour cells. Whilst only 30% of treatment-naïve tumours exhibited PDL1 expression, this was increased to 53% post-treatment. Furthermore, 63% of the negative treatment-naïve tumours acquired PDL1 expression after treatment. Comparable effects were observed *in vitro* with PDL1 expression induced by cisplatin treatment in PDL1-negative ovarian cancer cell lines⁶¹⁷. This is supported by preclinical studies that demonstrate that cisplatin treatment leads to an upregulation of PDL1 expression human and mouse ovarian cancer cell lines *in vitro* and *in vivo* via the activation of the cGAS and STING proteins in 2F8 ovarian cancer cells. Co-treatment of cisplatin and anti-PDL1 therapy promotes anti-tumour response to chemotherapy *in vivo* and enhanced survival in a platinum-resistant model. This coincides with increased CD8⁺ T cells, which appears to mediate anti-tumour response⁶¹⁸.

Interestingly, analysis of ovarian cancer samples in another study demonstrates that tumours that responded well to neoadjuvant therapy exhibited a more pronounced activation of CD4⁺ T cells with enhanced IFN γ expression and anti-tumour Th1 phenotypes, and decreased FoxP3⁺ Tregs. Whilst neoadjuvant chemotherapy did not affect the abundance of CD8⁺ T or memory T cells, a high proportion of the T cells in the less responsive tumours in the exhibited an upregulation of PD1, CTLA4 and PDL1 after neoadjuvant chemotherapy. Proinflammatory cytokines were also decreased after chemotherapy. Together this indicates chemotherapy may enhance the anti-tumour T cell-mediated immune response, but is hindered by the upregulation of immune checkpoint. Co-treatment of chemotherapy with immune checkpoint inhibitors may be therapeutically beneficial⁶¹⁹. This may suggest why we do not observe any changes in the abundance of T cells between untreated and chemotherapy-treated bone metastasis as instead there may be alterations in the checkpoint molecules. It would therefore be valuable to investigate the expression of the immune checkpoint molecules in the immune populations during chemotherapy treatment of breast cancer bone metastasis in our model.

Chemotherapy can also select for cancer cells with an immune evasive phenotype. TNBC cells treated with carboplatin, doxorubicin, gemcitabine or paclitaxel induces expression of CD47⁺ CD73⁺ PDL1⁺ breast cancer cells. CD47 evades the immune system by killing macrophages, whereas the CD73 and PDL1 mediate immunosuppression by killing cytotoxic T cells, inducing recruitment of MDSCs and altering the phenotype of dendritic cells. Together, the enrichment of breast cancer cells with this phenotype results in an immunosuppressive, pro-tumorigenic environment. This chemotherapy-induced effect was dependent on HIF, and therefore HIF inhibition improved chemotherapy efficacy⁶²⁰.

Another study demonstrates that inhibition of PDL1 in breast cancer cells during doxorubicin treatment decreases the apoptotic effect of chemotherapy. This is indicative of a protective role of PDL1 expression that prevents chemotherapy-induced apoptosis. Interestingly, doxorubicin treatment of the breast cancer cells resulted in a redistribution of PDL1 from the surface to the nucleus where it continued to exert tumour-promoting effects both *in vitro* and *in vivo*. Moreover, doxorubicin induced more prominent effects than daunorubicin whereas other chemotherapeutic agents, including docetaxel and cisplatin induced did not induce these effects suggestive of a specific response to each chemotherapy type. Although the effects on the TME were not examined, this study suggests a novel, unidentified role of PDL1 beyond its function in inhibiting T lymphocytes⁶¹⁵.

Other immunosuppressive molecules outwith the classical immune checkpoint molecules, such as Tim4, are also induced during chemotherapy treatment. Tim4 recognises and internalises fragments of apoptotic cells in order to prevent an immune response and is often upregulated in the TME in order to impair immune attack against the cancer. For example, in a model of B16-melanoma, Tim4 is highly expressed on intratumoural myeloid cells, including TAMs and dendritic cells. Moreover, in response to chemotherapy treatment, DAMPs released from chemotherapy-damaged tumour cells induces further Tim4 expression. It functions to repress the immune response that is activated by chemotherapy-induced apoptotic cancer cells. By uptaking and removing the apoptotic cells from the TME, antigen presentation of these cells is reduced and therefore the cytotoxic T-cell mediated response is impaired. This prevents destruction of the tumour and therefore permits tumour growth resulting in chemotherapy resistance⁵¹⁴.

5.3.2.1.3 Secretion of chemoprotective molecules

In response to chemotherapy, TAMs can also secrete factors that directly act upon the tumour cells to protect from chemotherapy-induced apoptosis. This includes macrophage-derived IL6, TNF α , EGF and CCL18⁶²¹. Co-culture experiments provide evidence that TAMs mediate resistance via this mechanism. *In vitro* co-culture of breast cancer cells with TAMs isolated from breast cancer tissue resulted in reduced cisplatin- and docetaxel-induced apoptosis. As this system does not consider other components of the TME, it demonstrates that resistance is at least partially due to TAM-derived factors that act directly only the tumour cells. This was supported by the reduced anti-tumour response observed in breast cancer xenografts derived from MCF-7 cells and TAMs isolated from breast cancer tissues, in comparison to tumour derived from cancer cells alone⁶²².

In pancreatic cancer, macrophages release IL1 β in response to chemotherapy treatment that protects the cancer cells from chemotherapy-induced apoptosis via the upregulation of COX2 and subsequent production of PGE₂ in the tumour cells. This is dependent on the ERK1/2 pathway, and could be attenuated using inhibitors of IL1 β , COX2 or the ERK1/2 pathway⁶²³. Similarly, upregulation of COX2 in tumour cells has been implicated in the chemotherapy resistance of other cancer models including neuroblastoma and thyroid cancer^{624,625}. TAM-derived TGF β also protects melanoma cells from chemotherapy-induced death both *in vitro* and *in vivo*. Chemotherapy, particularly temozolamide, accelerated the recruitment of these tumour-promoting macrophages to the melanoma resulting in a rapid appearance in the TME where they exert these effects and further enhance tumour progression⁶²⁶.

In the murine model of PyMT-driven breast cancer, paclitaxel treatment induced an infiltration of TAMs, which express tumour-promoting cathepsin proteases, particularly cathepsin B and S. Cathepsin-expressing macrophages protected against taxol-induced tumour cell death in *in vitro* co-culture experiments, which could be completely reversed using cathepsin inhibitors. Similarly, co-treatment with paclitaxel and cathepsin inhibitors sensitised mammary tumours to chemotherapy and significantly enhanced the efficacy of chemotherapy in both primary tumours and lung metastasis. Comparable effects were also observed with in other chemotherapy types that display different mechanisms to mediate cell death, including etoposide and doxorubicin treatment. This suggests that the recruitment of cathepsin-secreting macrophages is induced by a variety of different stimuli. Whilst the downstream effects of cathepsins

were not identified, the fact the cathepsin inhibition prevented chemotherapy-induced cancer cell death in culture with macrophage-derived conditioned media indicates that cathepsins regulate their effects by at least partially acting directly on tumour cells⁶⁰⁰. Macrophage-derived cathepsin B and S also promotes growth, angiogenesis and invasion in mammary and pancreatic cancer, and may also induce similar effects after chemotherapy treatment⁵²⁰. Furthermore, chemotherapy also stimulates the release of cathepsin B in MDSC. Both gemcitabine and 5-fluorouracil induces cathepsin B that in turn activates the inflammasome and release of IL1 β from the MDSCs. MDSC-derived IL1 β subsequently induced secretion of IL17 from CD4⁺ T cells resulting in immunosuppressive phenotype, promotion of tumour growth and subsequently hindered anti-tumour efficacy of chemotherapy in several cancer models including thymoma, lewis lung carcinoma, melanoma and breast cancer⁶²⁷. Myeloma cells exhibited protection against melphalan- and dexamethasone-induced apoptosis via direct cell-cell contact with TAMs. Interactions between P-selectin glycoprotein ligand 1 (PSGL1) and ICAM1 activate Src and Erk1/2 and suppressed caspase activation induced by chemotherapy. Inhibition of either molecule hindered the repressive effect of the TAMs both *in vitro* and *in vivo*, and resulted in chemotherapy sensitivity^{628,629}.

TAMs also enhance tumour cell survival and chemotherapy resistance by providing signals that promote the generation and survival of CSCs. In both lung and colon cancer, TAMs release milk fat globule-epidermal growth factor 8 protein (MFG-EGF8) that protects from cisplatin-induced cell death by inducing a CSC-like phenotype⁶³⁰. Similarly, macrophage depletion in pancreatic cancer decreases the number of CSCs and STAT activation in remaining CSCs, resulting in improved anti-tumour response⁵⁹⁹. Chemotherapy also promotes the generation of CSCs independent of TAMs. Analysis of matched biopsies from breast cancer patients before and after chemotherapy treatment demonstrates that chemotherapy-treatment increases CSCs and TGF β signalling, which contribute to re-growth of tumours. Preclinical work demonstrates that paclitaxel-induced expression of TGF β and IL8 and subsequent CSC (CSC) expansion in breast cancer cell lines can be prevented by TGF β type I receptor kinase inhibitors, which also prevent recurrence *in vivo*⁶³¹. In a model of ovarian cancer, a single treatment of cisplatin or paclitaxel causes an enrichment of CSCs. In comparison to naïve cells, chemotherapy-treated cancer cells injected into the intraperitoneal cavity displayed increased proliferation, increased oncogenic phenotype, and increase epithelial marker (E-cadherin), which is characteristic of a higher metastatic potential.

Together, these effects permitted increased tumour growth and higher tumour burden *in vivo*⁶³².

Macrophages also secrete molecules that inhibit chemotherapy response by competing with the chemotherapeutic drug during uptake and drug metabolism. In response to gemcitabine, highly abundant TAMs in pancreatic cancer exhibit alternatively activated metabolic status where they utilise oxidative metabolism to synthesise pyrimidines from glucose. These pyrimidines are subsequently released of which deoxycytidine directly competes with gemcitabine at the level of both drug uptake and metabolism. This prevents the uptake and incorporation into the cell, respectively, and thus reduces the efficiency of the chemotherapy⁶³³. Likewise, TAMs upregulate the expression of multi-drug resistance 1 and glucosylceramide proteins during cyclophosphamide, methotrexate and 5-fluorouracil treatment. These are responsible for efflux of chemotherapeutic agents from the tumour cells and therefore promoted resistance. CSFR1 inhibition reversed these effects resulting in reduced expression of the efflux proteins and subsequently an improved therapeutic response.

5.3.2.1.4 *Inhibition of mitotic and DNA damage*

TAMs can also suppress the efficiency of anti-mitotic chemotherapies, including taxol-based chemotherapy by inducing a process known as mitotic slippage to prevent cell death. Taxol-based chemotherapies induce their cytotoxic effects by disrupting the microtubule function during mitosis, resulting in mitotic arrest and subsequently cell death. In the PyMT breast cancer model, TAMs, particularly a MHC^{low} population, are recruited to paclitaxel-treated tumours where they produce unidentified secreted factors that promote a reduced duration of mitotic arrest and instead the tumour cells enter mitotic slippage. This results in survival and progression of the cell cycle, and therefore chemotherapy resistance. TAM depletion via the administration of CSFR1 inhibitor, BLZ945 prevented these effects, increasing paclitaxel-induced DNA damage signalling and subsequently cell death. Chemotherapy treatment of tumour cells cultured with MHC^{low} bone marrow-derived macrophages, but not MHC^{high} macrophages, results in the increased cells undergoing mitotic slippage and persistence of tumour cells compared to tumour cells cultured alone. This demonstrates that MHC^{low} macrophages directly suppress the ability of paclitaxel-based chemotherapies to induce genotoxic stress and cell death via the secretion of soluble factors. Similar effects were also observed with other chemotherapeutic agents, including carboplatin, doxorubicin, etoposide and gemcitabine, although to a lesser extent. Moreover, these effects were

dependent on MAPK/ERK kinase (MEK) signalling as MEK inhibitors prevented the protective effect of macrophages⁵⁵⁷. This is the first direct evidence to demonstrate the TAMs also modulate the molecular mechanisms involving the cytotoxic chemotherapy-induced cell damage. However, it is also supported by a previous study that demonstrates that paclitaxel-treated fibrosarcoma cells survived longer after mitotic slippage and are less apoptotic in the tumour *in vivo* compared to tumour cell cultured alone *in vitro*. Cancer cells cultured *in vitro* display prolonged cell cycle arrest and undergo apoptosis after treatment of anti-mitotic chemotherapies, whereas xenografts established *in vivo* from the same cancer cells arrest for shorter periods from where they enter mitotic slippage and survive. This further illustrates the influence of the TME in preventing apoptosis by inducing mitotic slippage, although the cell type was not identified⁶³⁴.

Macrophages also interact with MSCs to regulate the mechanisms that protect cancer cells from DNA damage during chemotherapy treatment of Lewis lung and colon carcinoma. In response to cisplatin treatment, MSCs secrete platinum-induced fatty acids, 12-S-keto-5,8,10-heptadecatrienoic acid and 4,7,10,13-hexadecatetraenoic acid (16:4(n-3)), which enter the circulation and activate F4/80⁺ CD11b^{low} macrophages in the spleen via activation of BLT2 signalling. This subsequently induces secretion of macrophage-derived lysophosphatidylcholines (LPCs) that protect the tumour cells from chemotherapy by reversing the cisplatin-induced DNA damage, alleviating cell cycle arrest and restoring cell proliferation. This results in induction of systemic chemotherapy resistance. Both macrophage depletion via liposome clodronate or blockade of BLT2 signalling inhibited the chemotherapy resistance, and could be reversed by treatment with LPCs. This response to chemotherapy was only observed in DNA-damaging, genotoxic chemotherapies, whereas paclitaxel and sorafenib, which are spindle poisons and tyrosine kinase inhibitors respectively, did not exhibit similar effects⁶³⁵. Interestingly the induction of the same MSC-derived fatty acids, 12-S-keto-5,8,10-heptadecatrienoic acid and 4,7,10,13-hexadecatetraenoic acid (16:4(n-3)), during cisplatin treatment is also responsible for chemotherapy resistance in ovarian cancer. Inhibition of the enzymes involved in the production of these platinum-induced fatty acids, including COX-1 and thromboxane synthase, prevents MSC-mediated chemotherapy resistance. These effects were again only observed with platinum analogs cisplatin, oxaliplatin or carboplatin, but not with 5-fluorouracil or irinotecan. Although the downstream effects of MSC-derived fatty acids were not observed, similar effects on macrophages may also occur in ovarian cancer¹²¹.

5.3.2.1.5 Angiogenesis

Angiogenesis also promotes resistance by facilitating tumour growth. The secretion of angiogenic growth factors and cytokines into the circulation is critical for the recruitment of the endothelial cells and precursors, of which many are upregulated in the TME during chemotherapy treatment, including VEGFA and PlGF⁶³⁶. A subpopulation of bone marrow-derived CD34⁺ VEGFR2⁺ endothelial cells is also enriched in the peripheral circulation of breast cancer patients receiving anthracycline and/or taxane neoadjuvant therapy, and was associated with an upregulation of VEGF, erythropoietin and angiopoietin2. These observations suggest that chemotherapy mobilises endothelial cells from the bone marrow to the tumour via the secretion of angiogenic factors, which contributes to neovascularisation and thus tumour growth⁶³⁷. Preclinical studies uncovered the molecular interactions involved in this process. In response to paclitaxel, but not gemcitabine, bone marrow-derived endothelial progenitors are released into the circulation and home to tumours, mediated by CXCL12 and VEGFR2. Targeting these factors inhibited these effects and enhanced the anti-tumour efficacy of chemotherapy⁶³⁸. Treatment of lewis lung carcinoma xenografts with paclitaxel, 5-fluorouracil or docetaxel induces the mobilisation of bone marrow-derived endothelial progenitors into the circulation, which home to the tumour, resulting in increased vasculature in the tumour and thus increase tumour growth. Notably, an increase in circulating endothelial progenitors was also detected in blood samples from patients receiving the same chemotherapies. These effects were not observed with not gemcitabine, cisplatin or doxorubicin indicating a chemotherapy type-specific effect. The mobilisation of endothelial precursors was due to a systemic release of CXCL12 and the subsequent effects on tumour vasculature and growth could be reversed by inhibition of VEGFR2 in an adjuvant setting with chemotherapy⁶³⁸. As the release of CXCL12 is systemic, similar effects may occur at metastatic sites.

Whilst these studies did not identify the source of the angiogenic factors, it has been recently demonstrated that cooperation between endothelial cells and immune cells, particularly macrophages, promote angiogenesis in tumours. It was observed in early studies investigating the mechanisms of chemotherapy resistance in ovarian cancer, small cell lung cancer and glioma that blood vessels were increased in chemotherapy-treated samples and was correlated with the abundance of macrophages⁶³⁹. It was subsequently demonstrated that paclitaxel-treated tumours exhibited an increased blood vessel density, which was reversed by CSF1R inhibition and improved

chemotherapy response¹⁴³. Together, this provided evidence that chemotherapy-induced TAMs enhance neoangiogenesis that promotes growth, resistance and pulmonary metastasis. Additional studies demonstrated that macrophages regulate the stability of blood vessels via secretion of macrophage-derived VEGF and TGF β . These are stored in the ECM and released by MMPs to promote angiogenesis. MMP9 is expressed by doxorubicin-recruited macrophages, and tumour established in PyMT;MMP9^{-/-} mice exhibit enhanced response to doxorubicin in comparison to control mice, indicating a potential mechanism mediating resistance. In addition, the release of MMP9 and therefore VEGF and TGF β also promotes vascular integrity and decreased leakiness thus limiting drug delivery to the tumours⁵⁹⁶. MMP2 and MMP12 have also been implicated in neoangiogenesis during TAM-induced resistance. Tumours treated with both CSF1R inhibitor and chemotherapies (cyclophosphamide, methotrexate and 5-fluorouracil) exhibited attenuated MMP2 and MMP9 expression, reduced angiogenesis and decreased growth by over 50% resulting in prolonged survival compared to tumours treated with chemotherapy only⁶⁴⁰. TAMs isolated from chemotherapy-treated breast cancer also display a TGF β - and IL4-dependent increase in chitinase 3-like protein 2 (Chi3L2), which promotes chemotaxis of myeloid cells that contribute to angiogenesis through VEGFA induction. Chi3L2 is associated with poor response and risk of distant metastasis⁶⁴¹.

More recently, it was demonstrated in a murine model of breast cancer and Lewis lung carcinoma that cyclophosphamide treatment induces that accumulation of a distinct population of CD206⁺ Tie2⁺ CXCR4⁺ TAMs at the perivascular site in a CXCL12-CXCR4-dependent manner. These secrete VEGFA to promote tumour vascularisation, resulting in relapse. Co-treatment with CXCR4 inhibitors and chemotherapy results in reduced tumour regrowth and enhanced chemotherapy sensitivity. A similar macrophage accumulation was observed in bone metastasis, although was not tested functionally⁶⁴². Similarly, a population of VEGFR3-expressing macrophages are enriched in paclitaxel-treated Lewis lung carcinomas and 4T1 breast cancer xenografts where they contribute to both angiogenesis and lymphogenesis. This was largely mediated by promoting the secretion of cathepsins, leading to an increased heparase activity, which in turn induced the expression of proangiogenic VEGF-C. This subsequently induced lymphangiogenesis and therefore tumour growth and metastasis. Inhibition of the VEGFR3/VEGF-C axis in chemotherapy-treated tumours attenuates these effects and restored sensitivity⁶⁴³. Interestingly, we also demonstrated that CD146 was increased in chemotherapy-treated bone metastasis, and may locate

to the blood vessels. Similar angiogenic effects may therefore also occur in bone metastasis and required further immunostaining of the blood vessels to confirm.

Taken together, these studies demonstrate that chemotherapy agents engage in a complex interaction with the immune cells, particularly of myeloid lineage. TAMs exhibit distinct responses between different chemotherapy and tumour types. The downstream mechanisms involved in TAM-mediated resistance include suppression of the T cell-mediated anti-tumour response, upregulation of immune checkpoints, secretion of chemoprotective factors, induction of CSCs, promotion of angiogenic factors, and prevention of pathways involved in chemotherapy-induced DNA damage, summarised in figure 5.3.1. Nevertheless, inhibition of chemotherapy-induced TAM infiltration enhances sensitivity to chemotherapy. It should be noted that in most studies there was little benefit of macrophage inhibition alone as a single therapy, and therefore clinical benefit may only be observed when administered in combination with chemotherapy. A clinical study of the use of CSF1R inhibitor PLX3397 in glioblastoma showed no efficacy as a single agent⁶⁴⁴, but demonstrated clinical benefits when given with standard treatments^{645,646}.

In our project, future studies are essential to determine the potential chemoprotective role of TAMs and T cells mediating doxorubicin resistance of bone metastasis. The identification of the signalling pathways involved in the potential tumour-promoting role of immune cells may permit the design of therapeutic strategies that combine chemotherapy with macrophage-inhibition approaches. These previous studies discussed studies not only offer insight into potential mechanisms, but also accounts for some of the observations made in chemotherapy bone metastasis. Firstly, we observe there is no significant change in myeloid or lymphocyte abundance in chemotherapy-treated bone metastasis. Other studies described here also observe this effect. Instead chemotherapy induces an activated phenotype in the TAMs and T cells that mediates their tumour-promoting effects. Secondly, these studies highlight that often a specific subset is responsible for the resistance, and therefore may not be identified in our quantification. Lastly, different subpopulations of immune cells exert opposing effects, and therefore depletion of the whole population may not reveal the effect of a population as the depletion of one subpopulation may counteract the other. This is particularly true to the CD8⁺ and CD4⁺ T cells. It is therefore important to identify any distinct immune cell populations. A more detailed characterisation of the immune cell populations is therefore vital, including the expression of immune checkpoint

molecules, to identify a distinct subpopulation of immune that may be involved in the effects of chemotherapy.

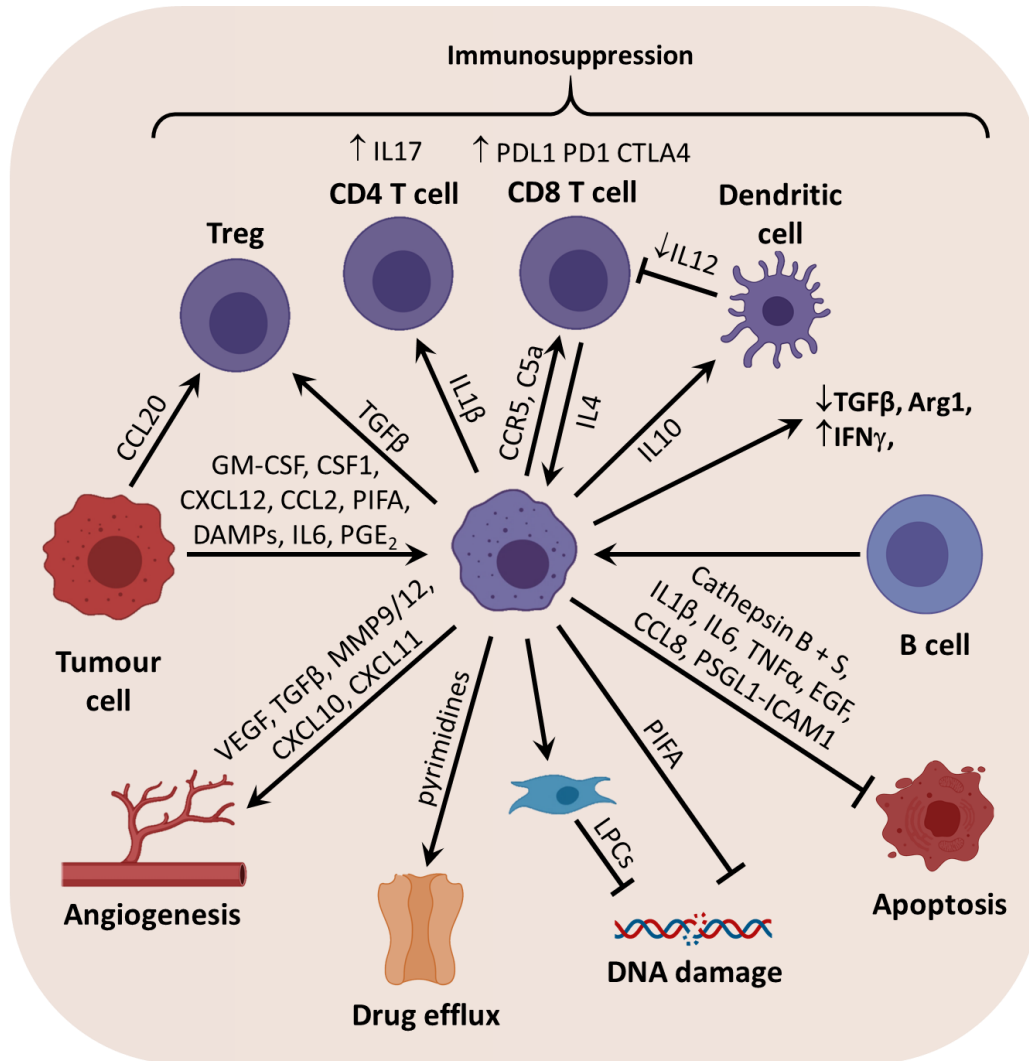


Figure 5.3.1: Role of macrophages in chemotherapy resistance. Macrophages induce resistance to chemotherapy by interacting with other immune cells to create an immune suppressive environment. They also secrete soluble factors that promote inhibit apoptosis and DNA damage response or promote drug efflux and angiogenesis. These effects are often dependent on activation of the macrophages by the tumour cells or chemotherapy treatment.

5.3.2.2 Mesenchymal cells

5.3.2.2.1 MSC-derived chemoprotective factors

The mesenchymal lineage within the TME is also an important mediator of the chemotherapy response and has been implicated in resistance in several cancer models. Chemotherapy treatment induces several changes in MSCs that subsequently

offer a protective effect against chemotherapeutic agents. Treatment of human bone marrow MSCs with chemotherapeutic agents, cytarabine, daunorubicin or vincristine, induced the upregulation of cytokines including IL6, FGF2 and TNF α . These chemotherapy-educated MSCs displayed enhanced tumour-promoting effects, including decreased tumour apoptosis during co-culture proliferation assays, in comparison to naïve MSCs⁵⁸⁷. Further studies demonstrate that chemotherapy alters the expression of additional molecular markers, including an upregulation of CD106, integrin α 11, MMP1 and MMP3 in MSCs that mediated chemotherapy response⁵⁶². Similarly, human MDA-MB-231 breast cancer cells cultured in MSC-derived conditioned media exhibited reduced response to doxorubicin compared to control tumour cells. These effects were dependent on IL8 and its depletion using neutralising antibodies confirmed a functional role¹²⁰. Notably, IL6 and serpin were also present in the conditioned media at high levels, but not tested functionally. The MSC-induced resistance was correlated with an increased expression of BCRP, which decreased the accumulation of intracellular doxorubicin. This was one of the first studies to demonstrate that expression of drug transporters, previously established as a significant component of the chemotherapy resistance, are mediated by the TME¹²⁰. Accumulating evidence also supports that IL8 acts in autocrine and paracrine manner to induce resistance in several human cancers, including colon cancer, ovarian cancer and melanoma, and is associated with increased expression of multidrug resistance-related genes, such as p-glycoprotein, as well as anti-apoptotic genes, Bcl2, Bcl_{XL} and XIAP, during cisplatin and paclitaxel treatment. Again, inhibition of IL8 reversed these effects^{647–649}.

Bone marrow-derived mesenchymal cells also promote doxorubicin resistance in multiple myeloma in an IL6-dependent manner. Co-culture of multiple myeloma cells with bone marrow-derived cells enhanced tumour growth in a range of doxorubicin concentrations compared to tumour cells alone. These effects were confirmed in several multiple myeloma cell lines, and similar effects were observed with cytarabine treatment. The effects were partially reversed by blocking IL6 or IL6R. Moreover IL6-IL6R inhibition had no effect on tumour cell growth when cultured alone confirming the role of stromal-derived IL6 in resistance⁶⁵⁰. IL6 has previously been shown to induce anti-apoptotic Bcl-2, which may mediate the resistance⁶⁵¹.

Platinum-based chemotherapies also induce the release of polysaturated fatty acids, 12-oxo-5,8,10-heptadecatrienoic acid (KHT) and hexadeca-4,7,10,13-tetraenoic acid

(16:4(n-3)) from MSCs, which promote resistance. Whilst the growth of colon and Lewis lung carcinoma xenografts is attenuated by cisplatin, intravenous injection of bone marrow-derived MSCs into tumour-bearing mice prior to chemotherapy treatment promoted resistance to cisplatin by preventing apoptosis. Similar effects were observed in human breast cancer xenografts indicating a potential intertumoral role. Relatively few MSCs were recruited to the tumour indicating they may exhibit systemic effects, and was confirmed using injection of conditioned media derived from chemotherapy-treated MSCs or MSCs at a site distant to the tumour, which induced similar effects. Further analysis demonstrates that platinum-based chemotherapies activate MSCs to induce a systemic release of the above fatty acids. Chemotherapy sensitivity could be restored by inhibiting the COX1/thromboxane pathway to prevent the formation of these fatty acids. The tumour-promoting effects of MSCs were also observed in other chemotherapies such as oxaliplatin and carboplatin, but not 5-fluorouracil and irinotecan, suggesting an effect specific to platinum-based therapies¹²¹.

Other molecular interactions have also been implicated in MSC-mediated resistance. MSCs protect MCF-7 breast cancer cells from apoptosis in a TGF β -dependent manner by utilising autophagy to recycle molecules and by inducing anti-apoptotic and pro-survival factors⁶⁵². Neuregulin 1 secreted from MSCs also activates PI3K/Akt signalling in colorectal cancer models to protect against chemotherapy-induced apoptosis⁶⁵³. Head and neck squamous carcinoma cells also exhibit increased survival and resistance to paclitaxel when co-cultured with bone marrow-derived MSC due to a attenuation of apoptosis, although the molecular interactions were not identified⁶⁵⁴.

Chemotherapy resistance is also regulated by CAFs. Fibroblastic, stroma-rich tumours such as pancreatic cancer often exhibit poor response to chemotherapy, indicating a role in the resistance. Borriello *et al.*⁴¹⁵ demonstrate that FSP⁺ FAP⁺ CAFs isolated from neuroblastoma tissue shared similar functional and phenotypic characteristics with bone marrow-derived MSCs and were thought to originate from the bone marrow. These CAFs enhanced tumour growth and chemotherapy resistance in both *in vitro* co-culture systems and *in vivo* tumour xenografts where tumours established from co-injection of CAFs and neuroblastoma cells displayed a reduced anti-tumour response to chemotherapy compared to neuroblastoma cells alone. CAFs increased the viability of neuroblastoma cells by stimulating proliferation and protecting the tumour cells from etoposide- and melphalan-induced apoptosis. Several cytokines, including CCL2, CXCL1, CXCL12, IL6, IL8 and serpin E1, were elevated in the CAF-derived

conditioned media, and thought to promote the protective effects, but were not tested functionally. However, the protective effects were dependent on JAK/STAT3 and MEK/ERK1/2 signalling in the neuroblastoma cells, and combined blockade of STAT3 and ERK1/2 sensitised the neuroblastoma xenografts to chemotherapy. The use of the *in vitro* co-culture demonstrates that CAF-derived factors can induce resistance by acting directly on the tumour cells. Furthermore, whilst the establishment of neuroblastoma-derived xenografts in immunodeficient mice suggests the effects of CAFs are independent on the immune system, a correlation between the abundance of these CAFs with TAMs was observed in treated neuroblastoma patient samples that were associated with poor response and prognosis. The cooperation between CAFs and TAMs may therefore be an alternative component important in the resistance mechanisms⁴¹⁵. CAFs also mediate resistance in lung cancer by secreting HGF, which inhibits paclitaxel-induced apoptosis via the upregulation of PI3K/Akt and GRP78 signalling. Using 3-dimensional co-cultures, Ying *et al.*⁶⁵⁵ demonstrated that CAFs inhibited paclitaxel-induced apoptosis and promoted tumour growth, which was attenuated in the presence of anti-HGF antibodies or PI3K or GRP78 inhibitors⁶⁵⁵. GRP78 is also upregulated in several chemotherapy refractory cancers, including breast, prostate, lung and haematological cancers indicating a potential role in resistance of other cancer types^{656–659}.

CAFs within pancreatic cancer secrete perlecan to promote resistance. Both tumour cells cultured with CAFs and tumours established from co-injection of CAFs with tumour cells exhibit decreased sensitivity and delayed response to gemcitabine and paclitaxel-based Abraxane. The secretion of perlecan into the also facilitates invasion into the surrounding matrix. Moreover, CAFs isolated from highly metastatic tumours induced a more prominent effect than those isolated from poorly-metastatic disease, and these differences were p53-driven. Inhibition of perlecan attenuates the effects of CAFs and enhances chemotherapy response to gemcitabine due to increased cell apoptosis and reduced proliferation resulting in prolonged mouse survival⁶⁶⁰. Therapeutic targeting of the protein synthesis mTOR/4E-BP1 regulatory pathway in pancreatic CAFs also prevents chemotherapy resistance. This pathway is highly activated in α SMA⁺ CAFs isolated from patient resections and contributes to enhanced protein synthesis and secretion of the CAF-secretome. Inhibiting this pathway during gemcitabine treatment reduces the CAF-secretome and induces synergistic sensitisation to chemotherapy⁶⁶¹.

CAFs also protect prostate cancer cells by attenuating DNA damage. Chemotherapy-induced DNA damage stimulates the expression WNT16B from CAFs in the TME of prostate cancer, and is mediated by NF κ B. Using laser-capture microdissection to isolate stromal and epithelial cells from prostate cancer for quantitative RT-PCR, it was demonstrated that WNT16B transcripts were increased sixfold in the stromal cells of chemotherapy-treated samples compared to untreated tumours. Similar effects were observed in breast and ovarian cancer. WNT16B acts in a paracrine manner to activate canonical Wnt signalling in tumour cells, which functions as a protective role against DNA damage induced by cytotoxic chemotherapies, mitoxantrone and docetaxel, *in vivo* resulting in proliferation and resistance⁶⁶². Similarly, co-culture of tumour cells with CAFs protected against apoptosis by counteracting the drug-induced oxidative stress, observed as reduced ROS. This mediated resistance to both doxorubicin and paclitaxel. CAF-derived conditioned media also prevented the accumulation of doxorubicin, but not paclitaxel, in prostate cancer cells and was associated with increase glutathione expression⁶⁶³.

Moreover, a distinct subpopulation of CAFs promotes chemotherapy resistance in breast and lung cancer. CD10⁺ GPR77⁺ CAFs correlated with chemotherapy resistance and poor survival cancer patients. It was confirmed that these CAFs were distinct from MSCs, which also express CD10, as flow cytometry demonstrated that CD146 was absent. These CAFs are not only resistant themselves, but they also promote tumour cell resistance as well as tumour formation. Survival of breast and lung cancer cells during chemotherapy treatment was significantly enhanced with culture of CD10⁺ GPR77⁺ CAFs due to secretion of survival factors, including IL6 and IL8 that protected CSCs from apoptosis. Moreover, CD10⁺ GPR77⁺-depleted CAFs did not induce these effects. Analysis of upstream effects demonstrates that C5a binds to GPR77, which activates NK-kB via p65 phosphorylation and acetylation, and results in expression of IL6, IL8, GPR77 and CD10 to maintain the abundance and tumour-promoting effects of CAFs. Patient-derived xenografts co-injected with this subpopulation of CAFs exhibit chemotherapy resistance, which can be abolished using anti-GPR77 inhibitory antibodies. This is important as therapeutic targeting of CAFs has detrimental adverse effects, and therefore specific targeting of a distinct population may overcome these issues⁵⁸⁸. This also highlights the requirement for further characterisation of the mesenchymal lineage in breast cancer bone metastasis.

Finally, chemotherapy also induces secretion of chemoprotective molecules from osteoblasts. In a model of breast cancer bone metastasis, whilst tumour-promoting Jagged1 is expressed autonomously on cancer cells, paclitaxel induces the expression of Jagged1 in osteoblasts, which exhibits pro-survival effects on tumour cells. Paclitaxel alone induces a slight reduction in bone metastatic growth in early time points, but resistance emerges in the later growth stage. In contrast, the co-treatment of Jagged1 inhibitory antibody, 15D11, with paclitaxel treatment acts in a synergistic manner to induces a significant, almost 100-fold reduction in tumour growth. Notably, 15D11 alone had little effect, with a superior therapeutic response observed in dual treatment, highlighting the importance of chemotherapy-induced Jagged1 expression⁶⁶⁴. Whilst we previously demonstrate that osteoblasts do not appear to play a role in bone metastatic growth, chemotherapy may induce similar soluble factors in osteoblasts to mediate resistance.

5.3.2.2.2 *Chemoprotective niches in the bone*

Within the bone, the previously described HSC niches composed of mesenchymal cells and osteoblasts provides anti-apoptotic signals and survival signals. Tumour cells residing within this protective niche are exposed to this signals and therefore promotes resistance^{28,75}. These signals include CXCL12, integrin $\alpha 4 \beta 1$ /VCAM1, fibronectin, thrombopoietin and angiopoietin1/Tie2 pathways, and are associated with poor response in patients⁷⁵. This provides a unique microenvironment for bone metastatic cells to grow in the presence of chemotherapy. Moreover, there is now increasing evidence that treatments, including chemotherapy, may further enhance this effect by inducing expansion of the niche. For example, in response to cytarabine and daunorubicin chemotherapy, leukaemic cells secrete cytokines including CCL3, TGF β and GDF15, which enhanced recruitment of Nestin⁺ Lepr⁺ NG2⁻ Nestin⁺ MSC to the niche. These recruited Nestin⁺ MSCs matured and transitioned to become α SMA⁺ in a TGF β -dependent manner. This therapy-induced expansion of niche subsequently promoted further tumour growth by releasing furin. These chemotherapy-induced effects were dependent on the tumour cells as they were not observed in non-tumour mice treated with chemotherapy⁶⁶⁵.

The CXCL12-CXCR4 signalling pathway is the most studied pathway involved in the chemoprotective HSC niche, particularly in leukaemia models⁷⁵. Acute myeloid leukaemia (AML) cells home and graft to the niche and is mediated CXCL12-CXCR4 signalling. This enhances the survival during chemotherapy treatment resulting in

resistance, which was attenuated in CXCR4-deficient mice compared to wildtype controls⁶⁶⁶. AML cell lines and patient isolates cultured with osteoblasts exhibited reduced apoptosis during chemotherapy treatment compared to cells cultured alone through soluble factor-mediated mechanisms. This demonstrates that osteoblasts protect AML cells from chemotherapy-induced apoptosis, whereas MSCs did not display protective effects and suggests the osteoblasts at the niche play a predominant role in providing anti-apoptotic signals⁶⁶⁷. Moreover, chemotherapy induces an upregulation of CXCR4 on the surface of AML cells, which further promoted stromal-mediated protection and resistance, and was reversed by CXCR4 inhibition⁶⁶⁸. CXCL12-CXCR4 signalling stimulates the several pro-survival pathways including MEK/ERK, JAK/STAT and PI3K/AKT, which regulate resistance⁷⁵. Furthermore, hypoxia, which is a characteristic of bone metastasis, induces that upregulation of CXCR4 in AML cell lines and may further promote resistance⁶⁶⁹. Additionally, hypoxia is associated with cell cycle arrest, upregulation of anti-apoptotic molecule XIAP, and chemotherapy resistance in AML, although the component of the TME involved was not identified⁶⁷⁰. Furthermore, MSC-derived CXCL12 also exhibits tumour-protective effects in ovarian carcinoma, and inhibition of CXCL12-CXCR4 signalling restores cytotoxic effects, further supporting a role in resistance⁶⁷¹.

Leukaemic cells also tightly adhere to osteoblasts via adhesions factors, such as integrins and cadherins, and through extracellular proteins, such as fibronectin and osteopontin. These interactions may function to home tumour cells to the chemoprotective niche⁷⁵. AML samples that display low expression of integrin $\alpha 4\beta 1$ have better prognosis and response rates compared to those positive for integrin $\alpha 4\beta 1$. Furthermore, AML cells cultured with stromal cells or fibronectin exhibited reduced apoptosis and were more resistance, which was reversed by integrin $\alpha 4\beta 1$ inhibition. This indicates a role of integrin $\alpha 4\beta 1$ -mediated interactions in chemotherapy resistance⁷⁵. Niche-derived thrombopoietin also mediates resistance by interacting with the receptor, myeloproliferative leukaemia protein (MLP). MLP expression in AML cells is associated with resistance⁶⁷². Osteoblast-derived thrombopoietin is also increased in AML patients compared to controls, particularly in patients with poor chemotherapy response⁷⁴. Preclinical studies show that thrombopoietin induces AML proliferation whereas its depletion reduced cell number in the niche⁶⁷³. AML cell lines were also protected from daunorubicin treatment when cultured with osteoblasts due to secretion of thrombopoietin. Together, this indicates that MLP-thrombopoietin interactions regulate AML resistance in the niche.

The niche has also been shown to promote resistance to chemotherapy by inducing tumour quiescence, or dormancy, and is also mediated by CXCL12-CXCR4 signalling. For example, chemotherapy-resistant AML cells home to and engraft to the endosteal osteoblast-rich niche where they are protected from chemotherapy-induced apoptosis due to quiescent phenotype adopted by the tumour cells⁵⁵⁵. In co-culture experiments, osteoblasts induced cell cycle arrest in cancer AML cells, which was attenuated by CXCR4 inhibition⁶⁷⁴. MSCs also mediate resistance by inducing tumour cell quiescence. MSC-derived CXCL12 acts upon leukaemia cells to stall the cell cycle and therefore induce quiescence during treatment. MSC-specific depletion of CXCL12 results in expansion of the leukaemic cells⁶⁷⁵. Osteopontin-mediated interactions with tumour cell at the niche also mediate resistance to chemotherapy in leukaemia by inducing a quiescent phenotype. Inhibition of osteopontin results in increases the population of cycling cells, which made them susceptible to chemotherapy⁷⁵. Other interactions established with the HSC niche also promote quiescence and dormancy in the bone metastatic cells including BMP, GAS6, integrin $\alpha 4 \beta 1$ or lack of Wnt and Notch^{183,676,677}. Interestingly, we similarly observe a reduction in proliferating cells in bone metastasis compared to MFP tumours suggestive of a potential quiescent or dormant phenotype, and may be mediated by similar mechanisms within the bone. These bone metastatic niches are absent in the MFP tumours that exhibit increased proliferation. It is therefore possible that the bone metastatic cells exist in or closeby to these niches that confers a dormant phenotype, and thus resistance in tumour cells.

It is clear that the mesenchymal lineage is activated by chemotherapy and subsequently exhibits tumour-promoting effects that induce resistance. This includes the secretion of chemoprotective factors that promote tumour cell survival, summarised in figure 5.3.2. Previous studies demonstrate that MSCs and osteoblasts within the HSC niche of the bone inhibit the chemotherapy response of leukaemia cells via the secretion of survival factors and induction of a quiescence phenotype. Breast cancer cells also reside within the HSC niche in bone metastasis where they may be exposed to similar chemoprotective effects. Similar to the studies described, we also identify a protective role of both MSCs and fibroblasts in co-culture with bone metastatic cells during chemotherapy treatment. Future studies should not only determine the molecular mechanisms involved in this interaction, but also validate the findings *in vivo*. This will involve a detailed characterisation of the mesenchymal compartment using flow cytometry and immunostaining to identify a distinct population enriched in chemotherapy-treated bone metastasis. Subsequently, cell depletion including Nestin⁺

MSC and SP7⁺ osteoblasts will confirm a functional role of these cells in chemotherapy resistance.

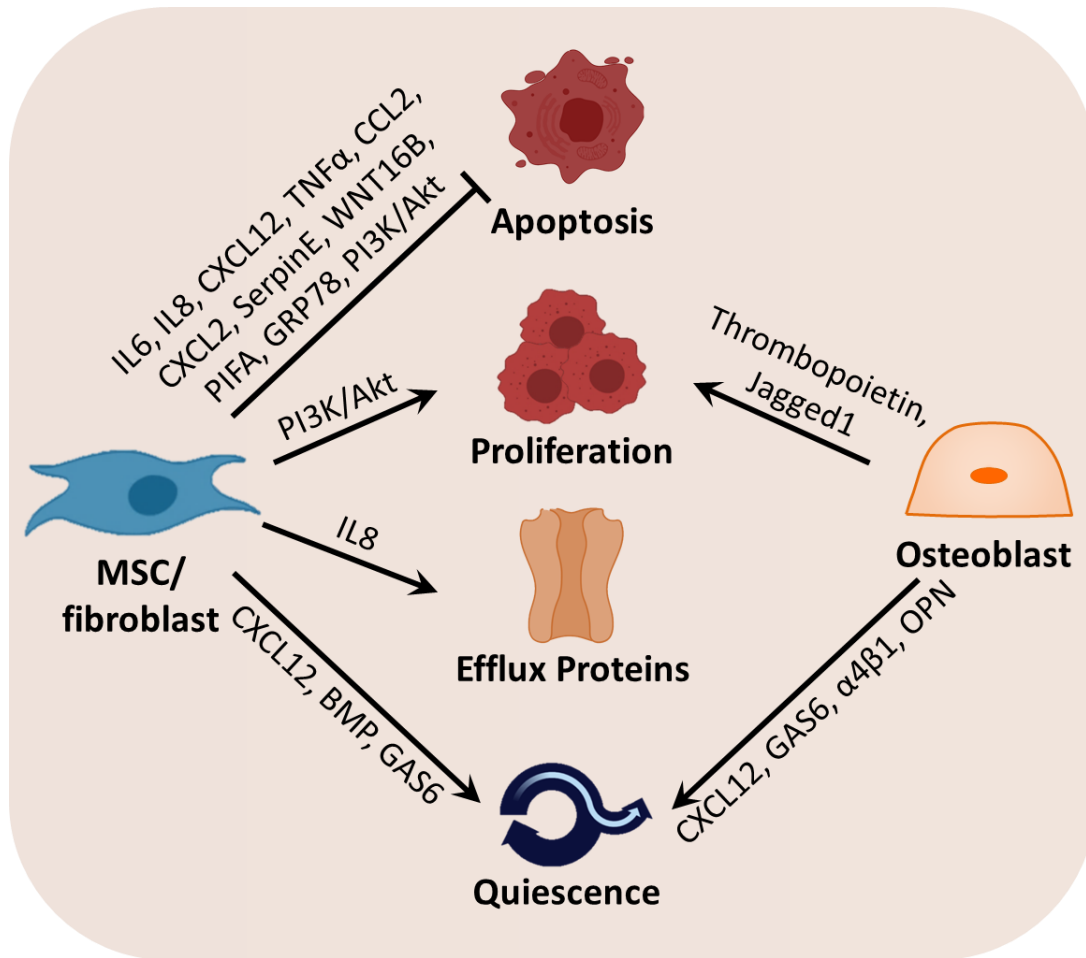


Figure 5.3.2: Role of the mesenchymal lineage in chemotherapy resistance. MSCs, fibroblasts and osteoblasts induce resistance through signalling pathways and survival factors that inhibit or promote apoptosis and proliferation, respectively. They also induce the expression of efflux proteins or a quiescent phenotype, rendering the cells resistance to chemotherapy treatment.

5.3.2.3 Vascular cells

Alternative to the mesenchymal and immune cells that we investigated, the vascular niche also mediates chemotherapy resistance via the secretion of angiogenic factors⁶⁷⁸. Chemotherapy resistant tumour cells associate with the vascular niche where they establish interactions with the endothelial cells. Within the bone, the perivascular niche has been found to provide a chemo-protective environment.

Integrin-mediated interactions established between the disseminated tumour cells and endothelial cells contribute to chemotherapy resistance to paclitaxel and doxorubicin. Driven by VWF, endothelial cells express integrin $\beta 1$ or $\alpha \beta 3$ that binds to VCAM1 on the tumour cells. Inhibition of this interaction sensitises the tumour cells to the treatment leading to reduced disseminated tumour cells in the bone and thus increased metastasis-free survival⁶⁷⁹. Integrin-VCAM interactions between the tumour cells and endothelial cells also regulate resistance to paclitaxel and doxorubicin in a murine model of mammary cancer. Targeting of endothelial-derived integrin $\beta 1$ and $\alpha \beta 3$ using neutralising antibodies sensitised the tumour cells to chemotherapy in an additive manner, highlighting their role in resistance. These effects of integrin inhibition were only observed after administration of chemotherapy suggesting that chemotherapy may enhance integrin expression first. Furthermore, endothelial-specific depletion of VWF has no effect on tumour growth in absence of chemotherapy, but induces apoptosis of tumour cells during chemotherapy treatment suggesting a role in the signalling pathway. Interestingly, the effects of the vascular niche were independent of the cell cycle status as the niche does not induce quiescence, but instead produces chemoprotective factors⁶⁷⁹.

In model of lymphoma, hepatocellular cancer and Lewis lung carcinoma, tumour associated endothelial cells secrete insulin growth factor binding protein-7 (IGFBP7), which inhibits the IGF1 secretion and expansion of IGF1R⁺ stem cell-like tumour cells. Conversely, chemotherapy reverses the suppression of stem cell-like tumour cells by repressing IGFBP7 secretion from endothelial cells. This therefore permits IGF1 activation of stem cell-like tumour cells that are resistant to chemotherapies, including doxorubicin. These tumour cells also subsequently express FGF4 that induces downstream effects involving FGFR1-ETS2, which further inhibits IGFBP7 creating a reinforcing feedforward loop. Disruption of this loop via FGF or IGF inhibition, establishment of tumours in IGFR^{-/-} mice or administration of IGFBP7 prevents resistance to several chemotherapies including doxorubicin, cyclophosphamide, daunorubicin and vincristine⁶⁷⁸.

Upregulation of Jagged1 in endothelial cells is also associated with chemotherapy resistance in B cell lymphoma. Jagged1 is stimulated by tumour-derived FGF4, and in turn, induces Notch2 signalling in the tumour cells to promote growth. Targeting of the FGF-Jag1-Notch2 paracrine loop via inducible deletion of *FGFR1* or *Jag1* or inhibiting Notch2 in a murine model of lymphoma reduces aggressiveness and enhances

sensitivity to doxorubicin chemotherapy⁶⁸⁰. The vascular niche may provide an alternative mediator of resistance in breast cancer bone metastasis that we did not investigate.

5.3.2.4 Osteoclasts

Another cell within the TME of bone metastasis that can be targeted to enhance chemotherapy response is the osteoclast population. Zoledronic acid is a bisphosphonate used to treat bone metastasis by inhibiting osteoclast-mediated bone resorption via the induction of apoptosis in osteoclasts. Their anti-tumour effects in the bone are reviewed by Clezardin⁶⁸¹. Co-treatment of zoledronic acid with chemotherapy also demonstrates synergistic effects to enhance chemotherapy response. In MDA-MD-231-derived breast cancer bone metastasis, Ottewill et al. recently demonstrated that sequential treatment of doxorubicin followed with zoledronic acid decreased tumour burden, particularly in the intraosseous, bone marrow region, and therefore resulted in significantly reduced osteolytic lesions compared to tumour treated with doxorubicin alone. This was mediated by an increased abundance in apoptosis and decreased proliferation of bone metastatic cells⁵⁸⁶.

Similarly, combined treatment of zoledronic acid with doxorubicin also improves sensitivity in bone metastasis of prostate and lung cancer^{563,682–684}. Paclitaxel resistance is also attenuated by zoledronic acid in breast and lung bone metastasis as well as other chemotherapies, including etoposide and cisplatin, by enhancing tumour cell death in a synergistic manner⁶⁸².

These studies demonstrate that osteoclasts may be a therapeutic target to enhance chemotherapy efficacy in bone metastasis. However, it should be noted that zoledronic acid does not only reduce osteoclastogenesis, but also has other significant impact on the TME of bone metastasis. For example, osteolysis during bone metastasis results in release of soluble factors from the bone that act on other cellular components of bone metastasis to enhance growth. Inhibition of osteoclastogenesis, which reduces osteolysis, therefore will prevent the release of these factors, which may prevent other downstream effects that mediate the sensitivity⁵⁸⁶. Moreover, it has recently been demonstrated that paclitaxel augmented osteoclast-mediated bone loss during treatment of bone metastasis, which could be inhibited by targeting the p38/MAPK/MK2 to maintain bone density⁶⁸⁵. Similarly, carboplatin treatment leads to cachexia and osteolytic trabecular bone loss in both healthy and metastatic bone⁶⁸⁶. As

osteoclastogenesis would contribute to the self-sustaining growth cycle observed in bone metastasis, these effects of paclitaxel may account for the chemotherapy-induced growth⁶⁸⁵. Together, these studies indicate that it may also be worthwhile to investigate the effects of paclitaxel and doxorubicin on the osteoclast population in bone metastasis as an alternative mediator of the chemotherapy response.

5.3.3 Chemotherapy-induced metastasis

Whilst the majority of tumour recurrences and metastases are resistant to treatment, it is recognised that chemotherapy displays clinical benefits to many patients with advanced cancer including restriction or slowing of further growth. However, there is now accumulating evidence to support that chemotherapy elicits unfavourable effects in the TME that stimulates further tumour progression and metastasis, defined as chemotherapy-induced metastasis. The alterations within the TME are induced either by direct effects of chemotherapy on the stromal and immune cells, or via chemotherapy-driven release of cytokines and growth factors from the tumour cell. Interactions are subsequently established between the chemotherapy-educated cells and the tumour cells that promote the tumour-promoting effects. Previous data demonstrating chemotherapy-induced metastasis focusses on the effect in the primary tumour that induces metastasis. This includes enhanced angiogenesis as well as invasion and extravasation, which promote the dissemination of tumour cells and subsequently initiation of metastasis⁵⁵⁶. We provide here the first evidence that chemotherapy, particularly paclitaxel, promotes the growth of already-established metastasis in the bone. Furthermore, these effects are independent of the presence of a primary tumour, but are dependent on the TME in the bone.

It should be noted that whilst chemotherapy can induce growth-promoting factors that act on the tumours, as described above, this usually only causes resistance where the tumour continue to grow at an equal or reduced rate compared to untreated controls. Chemotherapy-induced tumour progression is defined when the cancer develops at an increased rate compared to untreated samples.

The concept of treatment-induced tumour progression is not novel. It was first reported in the 1940s when it was observed in pre-clinical studies and several clinical cases that those receiving treatments exhibited increased metastasis. Further investigations in chemotherapy-induced metastasis occurred in the 1970s in a murine model of sarcoma lung metastasis where pre-treatment of mice with cyclophosphamide increased the

number of lung metastasis by up to 100-fold in a dose-dependent manner⁵⁵⁶. Although the mechanism was not investigated, the researchers concluded that this was not related to the immunosuppressive effects of chemotherapy. Interestingly, the effects of a single chemotherapy injection occurred as early as 2 hours after administration and lasted up to 8 weeks⁶⁸⁷. A similar study at the time demonstrated a comparative effect in mouse osteosarcoma where cyclophosphamide also increased the pulmonary metastasis⁶⁸⁸. More recently, Wu *et al.*⁶³⁹ demonstrated that cyclophosphamide enhances the tumour growth and metastasis in human xenograft models of ovarian cancer, small cell lung cancer and glioma. Pre-treatment 24 hours before inoculation with the tumour cells resulted in enhanced tumour growth and final volumes in all models, but also increased lung cancer bone metastasis and brain metastasis after intra-arterial injection⁶³⁹. However, it is not until recently that the cellular and molecular mechanisms involved in the treatment-induced tumour progression and metastasis are being investigated.

Increasing evidence demonstrates that the response of the TME and the host tissue to chemotherapy treatment can counteract the anti-tumour activity by promoting metastasis¹⁰⁸. During metastasis, cancer cells undergo the well-defined, yet intricate, step-wise process known as the metastatic cascade. Research shows that chemotherapy promotes many of the crucial stages of the metastatic cascade often through manipulation of the tumour microenvironment, thus increasing the risk of metastasis. Chemotherapy also induces a systemic inflammatory response that promotes metastasis. It is also important to highlight that other treatments, including surgery and radiotherapy, have also been shown to promote tumour progression and metastasis. This will not be discussed here and is reviewed by Ebos⁶⁸⁹, but it may also be valuable to determine the effects of other treatments used in the clinic on bone metastatic growth in future experiments.

5.3.3.1 Chemotherapy-induced inflammation

The TME is similar to 'a wound that never heals' exhibiting similar characteristics to an inflammatory response. Often cancer treatments, and even diagnostic biopsies, can create an inflammatory response either at the local or systemic level. This includes secretion of inflammatory cytokines and recruitment of inflammatory cells, predominantly derived from the bone marrow, which together promote increased growth, angiogenesis, cancer cell invasion and thus metastasis. Chemotherapy-enhanced inflammation and recruitment of tumour-promoting inflammatory cells is a

common feature across many types of chemotherapy with several roles described in different cancer types. The inflammatory mechanisms resulting in chemotherapy-induced metastasis are reviewed by Vyas et al⁶⁹⁰. Here, we will focus on the effects of the inflammatory response on the growth of already established tumours as these are most applicable to the paclitaxel-induced bone metastatic growth.

Inflammatory-mediated responses during chemotherapy were previously thought to be responsible for the anti-tumour responses by enhancing tumour-antigen recognition by T-cells. For example, anthracyclines, particularly doxorubicin, induce type I IFN release from tumour cells via activation of TLR3 and triggers autocrine and paracrine signalling networks that result in CXCL10 release. Cancer cells lacking TLR3 or IFN α failed to respond to chemotherapy, and this effect could be rescued by supplying type I IFN or CXCL10 exogenously. This demonstrates that IFN α -induced CXCL10 sensitises tumours to chemotherapy⁶⁹¹. Tumour cells also release ATP whilst undergoing cell death that recruits myeloid cells into tumours and stimulates the differentiation of dendritic-like cells. These antigen presenting cells engulf the tumour antigens and present them to the T lymphocytes, therefore creating an immune response against the tumour cells⁵⁹⁴. However, in contrast, the inflammatory response may in fact induce cancer progression and metastasis. Chemotherapy can result in the release of pro-inflammatory factors from both the tumour cells and the tumour microenvironment, which can have multiple roles in promoting metastasis. These can act directly on the cancer cells to enhance different aspects of tumour progression, or can also recruit immune cells that influence the cancer cells.

Chemotherapy, including 5-fluorouracil and gemcitabine, promotes pro-inflammatory response at a local and systemic level, including the release of IL1 β , IL6, IL8, IL17 and GM-CSF from myeloid and CD4⁺ T cells, which results in tumour progression. In response to gemcitabine, CAFs also secrete pro-inflammatory factors including IL6, IL8, CXCL1, CXCL6, ICAM1 and osteopontin, which together were termed as a senescence-associated secretory phenotype, and were mediated by MAPK signalling. Their release leads to increased proliferation *in vitro* as well as increased tumour growth *in vivo*. Inhibition of the MAPK signalling reversed these effects indicating a therapeutic strategy⁶⁹².

Chemotherapy-induced cell death also initiates a pro-tumour response as tumour cells undergoing apoptosis due to the cytotoxic effects of chemotherapy release acute

inflammatory cytokines and prostaglandins, which promote metastasis⁵⁵⁰. Debris and other fragments from apoptotic cells during paclitaxel treatment of breast cancer contributes to increased incidence and burden of pulmonary and lymph node metastasis in a TLR4 dependent manner. The release of debris and fragments of apoptotic cells during paclitaxel treatment activates TLR4 on the breast cancer cells, which results in increase in inflammatory cytokines including IL6, IL10, IL1 β and IL8 in the TME. At a local level, this contributes to increased blood vessel and lymphatic vessel formation, which facilitates further growth as well as extravasation into the circulation. Systemically, the inflammatory cytokines recruit Ly6C⁺ and Ly6G⁺ myeloid progenitor cells to the primary tumour and distant metastasis. Together, this drives further growth and a pro-metastatic phenotype therefore favouring primary tumour progression and metastasis to the lungs. Blocking paclitaxel-induced inflammation via TLR4 inhibition may improve clinical outcomes⁵⁵². Chemotherapy-induced DNA damage also plays an important role in inducing a growth-promoting inflammatory response. In a resistant model of Burkitt's lymphoma, doxorubicin-induced DNA damage induces IL6 and Timp1 release from the endothelial cells in the thymus mediated by p38 MAPK stress response. This protects that tumour cells by inducing expression of anti-apoptotic gene Bcl_{XL} and promotes their proliferation, and could be overcome by inhibition of IL6 and Timp1. Similar effects were also observed in human liver cancer cells and in other treatments such as paclitaxel and radiation⁶⁹³.

Recent evidence demonstrates that targeting the chemotherapy-induced inflammatory response reduces recurrence and metastasis growth thus increasing survival in several cancer models. Pre-operative treatment with the non-steroidal anti-inflammatory drug ketorolac reduces growth and metastasis by dampening inflammation via the inhibition of the COX-1/TXA₂ pathway, whereas Resolvins eliminate micrometastasis by inducing the resolution of inflammation. Both exhibit anti-tumour activity in a synergistic manner via the induction of an anti-tumour T cell response characterised by reduced FoxP3⁺ T cells, exhausted T cells and Ly6G⁺ neutrophils, and increased tumour-specific T cells, CD4⁺ T cells and CD8⁺ T cells. Together, this results in elimination of tumour cells⁶⁹⁴.

5.3.3.2 Induction of the metastatic cascade

During metastasis, cancer cells undergo the well-defined, yet intricate, step-wise process known as the metastatic cascade. There is now accumulating evidence to demonstrate that chemotherapy treatment of the primary tumour increases the risk of

metastasis by promoting many of the crucial stages of the metastatic cascade often through manipulation of the TME.

Firstly, chemotherapy enhances migration of cancer cells, an important step during the initial stages of the metastatic cascade. This is through the promotion of several mechanisms including the acquisition of a migratory phenotype via the EMT, the production of factors that act as chemoattractants, and the secretion of molecules such as MMPs that degrade the ECM. Together, they enhance the invasion of cancer cells and thus metastasis. Chemotherapy induces the EMT in several cancer models including lung, breast and ovarian cancer with treated cancer cells exhibiting mesenchymal-associated genes, an enhanced migratory phenotype and an increased metastatic potential^{695,696}. In a human lung cancer xenograft, apoptotic cells during chemotherapy treatment release HMGB1-containing nucleosomes, which are also increased in the circulation of lung cancer patients receiving chemotherapy treatment, and are associated with cancer invasion and advanced stage. The HMGB1-containing nucleosomes mediate cancer cell invasion and metastasis by binding TLR4 and TLR9. Inhibition of HMGB1 prevents these chemotherapy-induced effects resulting in decreased metastasis suggesting a therapeutic target⁶⁹⁷. Similarly, paclitaxel treatment of both breast cancer cell line MDA-MB-231 and glioblastoma cell line UI87VIII results in enhanced EMT, invadopodia formation, and invasion and migration due to activation of the CDK5 pathway. Inhibition of the CDK5 pathway decreased downstream targets including FAK in the cancer cells and therefore prevented the paclitaxel-induced EMT. This coincides with reversal of paclitaxel-induced pulmonary metastasis in a murine model of breast cancer after CDK5 treatment, suggesting that targeting the pathway may provide a neoadjuvant therapy in combination with paclitaxel treatment⁶⁹⁶. Ovarian cancer cells also undergo the EMT after paclitaxel, doxorubicin and cisplatin treatment, and exhibited an increased metastatic potential, which contributes to the chemotherapy-induced metastasis. CXCR4 antagonists in combination with chemotherapy reversed this effect indicating a therapeutic potential⁶⁹⁸. Cisplatin treatment also promotes a mesenchymal-like phenotype in a model of osteosarcoma in a Snail dependent manner, which leads to increased pulmonary metastasis. Snail knockdown not only prevented chemotherapy-induced EMT and thus reduced pulmonary metastasis, but also increased the sensitivity to cisplatin^{695,699}. The EMT is partly regulated by the expression of mRNAs, including miRNA488, which suppresses the EMT. Chemotherapy treatment impairs miRNA488 signalling in a NF- κ B-dependent manner, which removes the EMT inhibition. Patients undergoing cyclophosphamide,

epirubicin and 5-fluorouracil chemotherapy treatment also exhibit downregulation of miRNA488 therefore may play a potential role in chemotherapy-mediated EMT induction⁷⁰⁰.

Chemotherapy can also increase migration by inducing the production of MMPs that mediate invasion through the matrix. Paclitaxel treatment enhanced metastasis of Lewis Lung carcinoma, which could be prevented by co-treatment with MMP9 inhibitor. As well as the induction of MMP9 from the bone marrow-derived cells that are recruited to the tumour, paclitaxel also induces the EMT, which results in an increase in migratory phenotype. It also enhances endothelial cell migration and vessel sprouting to the tumours. Together, the increased tumour migration and blood vessels permits increased tumour invasion into the circulation, and thus enhanced metastasis⁷⁰¹. This is further supported by enhanced response during doxorubicin treatment in MMP9-null mice⁵⁹⁶. Another study demonstrated that cyclophosphamide treatment of lung adenocarcinoma xenografts enhanced metastasis to the lung. This coincided with an increased in MMP2 suggesting a potential role, but was not tested functionally⁷⁰². Chemotherapy can also degrade the ECM by inducing the release of heparanase-loaded exosomes. Surviving myeloma cells after bortezomib, carfilzomib or melphalan treatment upregulate heparase, which is secreted in exosomes in response to chemotherapy treatment. Heparase is found on the exosome surface therefore was able to degrade heparin sulphate within the ECM, resulting in ECM remodelling and thus facilitating increased migration. In addition, macrophages exposed to the exosomes express TNF α , which is known to promote growth of myeloma, and exhibit increased migration. Although the effects of the chemotherapy-induced TAM recruitment were not investigated, it is possible that they lead to chemotherapy resistance and recurrence⁷⁰³.

Chemotherapy also promotes metastasis by facilitating intravasation from the primary tumour into the circulation. Whilst direct clinical evidence for chemotherapy-induced release of tumour cells into the circulation is scarce, several clinical studies demonstrate that patients exhibit increased CTCs after chemotherapy treatment. In a cohort of NSCLC, SCLC and breast cancer patients, approximately one third of the patients exhibited CTCs after treatment when they had none before, and 1 in 10 exhibited an increase in CTCs after treatment⁷⁰⁴. Preclinical models have given insight into the mechanisms resulting in increased CTCs, including intravasation. One research group recently used mouse models of breast cancer, including the PyMT model and patient-

derived xenografts, to demonstrate that whilst paclitaxel, doxorubicin or cyclophosphamide successfully reduced the growth of primary tumours, it also increased the risk of metastatic dissemination. Neoadjuvant paclitaxel promoted the assembly of microanatomical structures around the blood vessels, termed as tumour microenvironment of metastasis (TMEM). TMEMs are composed of Tie2^{hi}, VEGF^{hi} perivascular macrophages, an endothelial cell and the intravasating tumour cell, which expresses actin-regulatory protein Mammalian-enabled (MENA). These structures act as a gateway for the tumour cells to intravasate into the circulation and are associated with an increase in circulating tumour cells. Notably, patients who have received neoadjuvant chemotherapy treatment exhibit increased circulating cancer cells in comparison to before treatment. Together, this supports that chemotherapy may help cancer cells escape from the primary tumour into the circulation thus increasing the risk of metastatic dissemination. Targeting the cellular-gateways via molecular inhibition of the Tie2 receptor with Rebastinib reduced the number of Tie2^{hi}/VEGF^{hi} macrophages and decreased the number of circulating tumour cells thus suppressing cancer cell dissemination. However, the effects on clinical outcomes including metastasis incidence and survival was not analysed⁵⁵⁴. This mechanism of TMEM is supported by Chang *et al.*⁷⁰⁵ who demonstrate in a model of breast cancer lung metastasis that paclitaxel treatment also increases the total vasculature volume, indicative of enhanced angiogenesis, and decreases pericyte coverage of the vessels in the primary tumour. Together this increases the vessel area for the tumour cells to escape leading to increased circulatory tumour cells and subsequently increased lung metastasis⁷⁰⁵.

After the intravasation and travelling through the circulation to the secondary site, tumour cells must colonise a niche in order to persist and survive. The formation of this pre-metastatic niche precedes the arrival of the tumour cells and both attracts the tumour cells to the secondary site and promotes their survival once there. Chemotherapy treatment can promote the formation of the pre-metastatic niche by directly altering the host cells at the secondary site. One of the first examples was described in an experimental model of pulmonary metastasis of breast cancer, colon cancer and melanoma where pre-treatment of cisplatin and paclitaxel prior to intravenous injection of tumour cells significantly enhanced lung metastasis due to the chemotherapy-induced expression of VEGFR1 on endothelial cells. This enhanced adhesion of tumour cells to the niche and thus colonisation. Anti-VEGFR1 inhibitory antibodies reversed this effect and reduced metastasis incidence indicating an effective therapy to prevent chemotherapy-induced lung colonisation by tumour cells⁷⁰⁶. The

release of bioactive lipids, S1P and C1P, from the bone marrow cells acts as a chemoattractant of rhabdomyosarcoma cells. Their release is increased in the bone marrow during chemotherapy treatment and enhanced migration, which was attenuated using inhibitors of the lipids. This suggests that chemotherapy can increase metastasis by inducing migration of tumour cells from the primary tumour to the metastatic site, including the bone. S1P and C1P is also involved in metastasis of lung, breast, prostate and liver cancer, and may therefore mediate similar effects⁷⁰⁷.

Treatment can also induce the mobilisation of bone marrow-derived cells, including endothelial progenitor cells and monocytes, to the secondary site from where they produce chemokines and other signalling molecules to recruit disseminated tumour cells to the niche⁷⁰⁸. Chemotherapy creates a pre-metastatic niche in the bone, which enhances metastasis in a prostate cancer murine model. Pre-treatment with cyclophosphamide prior to intracardiac injection of prostate cancer cells significantly enhanced subsequent bone metastasis growth and burden. This was due to expansion of myeloid lineage cells, including monocyte and neutrophils, in the bone marrow as well as upregulation of VEGFA, IL6 and CCL2, which are involved in myeloid cell proliferation and differentiation. This tumour-promoting effect was reversed by CCL2 inhibition, but not IL6 inhibition, where neutralizing antibodies significantly reduced bone metastasis. This effect was not observed using docetaxel suggesting it may be chemotherapy-type specific⁷⁰⁹. Bone marrow-derived cells are also recruited to the pre-metastatic niche of breast cancer lung metastasis. Whilst paclitaxel reduced the growth of the primary tumours, it induced metastasis by inducing CCL2-mediated recruitment of inflammatory monocytes to the pre-metastatic niche resulting in increased colonisation and thus metastasis. These effects were dependent on host-cell Atf3, which binds to the CCL2 promoter, as the effects were abolished in *atf3*-KO mice⁷⁰⁵.

Several studies have also demonstrated that treatment can indirectly promote the pre-metastatic niche formation by causing the release of signalling molecules from primary cancers that act upon the secondary site in preparation for the arrival of the cancer cells, analogous to sending out fertilisers to prime the soil to help the seed grow⁶⁸⁹. Recently, Keklikoglou *et al.*⁷¹⁰ demonstrated that both paclitaxel and doxorubicin enhances spontaneous and experimental pulmonary metastasis in murine mammary tumour models. In response to paclitaxel and doxorubicin treatment, annexin A6-rich extracellular vesicles are released from the primary tumour, which are also detected in the circulation of breast cancer patients undergoing neoadjuvant chemotherapy. These

act on the pulmonary host cells to induce CCL2, CSF1, CXCL12 and angiopoietin-2 expression and Ly6C⁺ monocyte expansion as well as promoting inflammatory endothelial cell activation. Together, this creates a favourable pre-metastatic niche for seeding and mediates the pro-metastatic activity of the chemotherapy treatment⁷¹⁰.

Finally, another study investigating the effect of chemotherapy on lung metastasis demonstrated that paclitaxel and carboplatin treatment of the primary cancer increased lung metastasis via multiple mechanisms. Chemotherapy-treatment induced pro-tumorigenic cytokines and angiogenic factors including CXCR2, CXCR4, S1P/S1PR1, PIGF, PDGF-BB and CXCL1 in the serum and primary tumour. This resulted into increased angiogenesis and decrease vascular integrity in the primary tumour, recruited bone marrow-derived cells to the secondary sites creating a receptive pre-metastatic niche, and directly promoted proliferation, migration and the EMT in the tumour cells. Together, this promoted lung metastasis, which could be attenuated by targeting these factors, predominantly inhibitors of the CXCL12-CXCR4 and S1P-S1PR1 axes. This highlights the complexity of the chemotherapy-induced effects on metastasis and resistance, and the involvement of multiple mechanisms.

Whilst many of these studies identify effects within the primary tumour that initiate components of the metastatic cascade, many of the effects are mediated by the activation of bone marrow-derived cells that are recruited to the primary tumour or the metastatic niche. Moreover, chemotherapy-induced inflammation is a systemic effect. Similar activation may therefore also occur during chemotherapy treatment of bone metastasis where bone marrow-derived cells are recruited to the tumour and exert alternative effects to promote growth.

5.4 Conclusion

Here we demonstrate that breast cancer bone metastasis is resistance to doxorubicin, whilst paclitaxel promotes further progression, defined as chemotherapy-induced growth. We also confirm that these effects were cell non-autonomous, and are dependent on the TME of the bone. We were unable to identify the exact cell type that mediates these effects, however, we provide evidence that the mesenchymal lineage may contribute, and should be further investigated. Research should also be performed to further investigate the role of the immune compartment. Whilst we show that the immune populations, particularly the macrophages and lymphocytes are not significantly changed in chemotherapy-treated metastases, previous studies have highlighted that specific subpopulations can be activated to mediate resistance. Experiments involving cell depletion of the immune populations were inconclusive due to issues with the adverse effects. Future research should therefore involve detailed characterisation of both the immune and mesenchymal compartment to identify potential distinct alterations and populations in the chemotherapy-treated bone metastasis, which can then be specifically targeted. This will overcome the issues regarding the adverse effects. This may give insight into a novel therapy that can be given in an adjuvant setting in combination with chemotherapy to prevent both chemotherapy resistance and chemotherapy-induced bone metastatic growth.

Whilst it is established that cancers, particularly metastases, display chemotherapy resistance, the paradox of chemotherapy-induced tumour progression and metastasis is an evolving field. Previous research has focused on the effects of chemotherapy treatment of the primary tumour on the early stages of the metastatic cascade. This includes chemotherapy-induced changes in the TME that promote extravasation and formation of the pre-metastatic niche, and subsequently spread and metastasis. We provide here evidence to suggest that chemotherapy treatment also exhibits tumour-promoting effects on tumour cells already at the metastatic site. To our knowledge, this is the first study that has demonstrated chemotherapy-induced growth of established tumours at the metastatic site, and is the most important finding in this chapter.

There is currently limited knowledge regarding the optimal management and clinical outcome of bone metastasis. Chemotherapy is often provided in combination with other options including endocrine therapy, molecular targeted therapy, or bisphosphonates, as well as radiotherapy and surgical interventions. These treatments are not only in

effort to restrict further growth, but are also an integral component of pain management. Chemotherapy is an aggressive and toxic treatment, and careful considerations regarding the risk-benefit balance must be deliberated. We suggest here that chemotherapy, at least as an individual treatment, may in fact promote bone metastasis rather than restrict growth; however, clinical trials are required to confirm this. Moreover, it is important to consider that at the time of diagnosis, disseminated cancer cells from the primary tumour are detected within the bone marrow of 15.5-30% and 13-72% of breast and prostate cancer patients, respectively, and can give rise to metastatic lesions. The presence of cancer cells within the bone marrow is associated with recurrence, reduced disease-free survival and therefore poor prognosis. It is therefore important to recognize the undesired tumour-promoting effects of chemotherapy on these DTCs and micrometastases already in the bone. These will be exposed to the chemotherapy during treatment of the primary tumour and may mediate their outgrowth into metastatic lesions.

Nevertheless, despite their undesirable pro-metastatic effects, current cancer therapies are often successful in eradicating primary cancer and remain to be a valuable, and certainly indispensable, element of cancer treatment and care. Further research into the effect of cancer treatments on metastasis is therefore necessary as well as a full understanding of why some patients respond fully, whereas recurrence occurs in others. By identifying the molecular mechanisms and signalling pathways involved in resistance and chemotherapy-induced progression, researchers can identify potential therapeutic targets that can be exploited. This may come in the form of an adjuvant therapy given alongside current treatments to prevent the undesired and opposing influences on cancer progression.

Chapter 6 Conclusion

The role of the TME in tumour progression in primary tumours has been extensively researched, whilst its contribution to bone metastasis remains to be elucidated. Here, we utilise a novel experimental model to investigate the contribution of the TME in breast cancer bone metastasis throughout each stages of its progression. Firstly, we demonstrate that macrophages are important in the early stage of bone metastasis. They mediate the tumour cell seeding and colonisation in the bone, and macrophage depletion results in significant delay in bone metastasis progression. We have also developed novel imaging techniques including an intravital imaging platform and tissue clearance protocol that will permit visualisation of tumour cell seeding to the niche within the bone in real time, and allow investigation into the effect of macrophage on this process.

We next determined that MSCs, particularly the Nestin⁺ population, plays a role in the growth of breast cancer bone metastasis. The depletion of Nestin⁺ MSCs results in an almost eradication of the metastasis in the bone, whereas bone marrow-derived MSCs promote growth of the bone metastatic cells *in vitro*. To our knowledge, this is the first evidence that nestin⁺ MSCs significantly contributes to the development of bone metastasis, or any other cancer type. Additionally we demonstrate that the CD146⁺ MSCs correlate with bone metastatic growth, and therefore further characterisation should be performed to determine if they overlap with the nestin⁺ MSCs or if they exist as a distinct population. Moreover, our preliminary data indicates that MSCs also induces recruitment and polarisation of macrophage into a TAM-like phenotype that has previously been shown to promote bone metastatic growth.

Finally, we investigated the response of breast cancer bone metastasis to chemotherapy treatment. Whilst bone metastases were resistance to doxorubicin, paclitaxel treatment induced further growth compared to untreated controls. This is clinically significant as patients often receive chemotherapy as part of palliative care to restrict the growth of bone metastasis. Moreover, a proportion of patients present with metastatic spread at the time of primary diagnosis, and therefore breast cancer cells that have often already disseminated to the bone will be exposed to chemotherapy

during the treatment of the primary tumour. These responses to chemotherapy were due to cell non-autonomous effects and dependent on the TME within the bone. It is therefore essential to identify the cellular mechanisms involved in the chemotherapy response, and whilst our initial investigations were inconclusive, future research should aim to identify the cell type by providing a detailed characterisation of the alterations in the TME in response to chemotherapy, which can then be tested functionally. Chemotherapy remains an integral component of anti-cancer treatment. The identification of the mechanisms involved in the response may present an opportunity to allow future development of a drug administered with chemotherapy in an adjuvant setting to prevent these undesirable effects.

In summary, we provide evidence that the TME plays an important role in the progression of breast cancer bone metastasis from early stage seeding and colonisation to late stage growth, as well as the response to chemotherapy. Future experiments should include the utilisation of the intravital imaging platform and tissue clearance technique to determine the role of the macrophages in the breast cancer seeding to the bone and identify the exact location of the seeding niche. These techniques may also permit the visualisation of the infiltration of stromal cells to the bone metastasis during growth and chemotherapy treatment. The cellular components of the TME should also be further characterised using immunostaining and flow cytometry analysis to determine the exact cell type or cell subset responsible for the tumour-promoting effects, including bone metastatic growth, chemotherapy resistance and chemotherapy-induced metastasis, as described throughout. Finally it is important to investigate the molecular mechanisms involved in mediating the pro-tumorigenic effects of the cells within the TME at each stage of breast cancer bone metastasis, including RNA-sequencing, qPCR and cytokine arrays, as well as depletion models and molecular inhibition or knockdown to confirm a functional role.

Collectively, this may offer potential therapeutic targets throughout the development of bone metastasis and offer novel strategies to treat this currently incurable disease. Treatments to prevent bone metastasis may involve inhibiting macrophage-mediated seeding, whilst targeting the MSCs during growth may provide a therapy for established bone metastasis. Furthermore, an adjuvant therapy against the TME to inhibit the undesirable effects involved in the chemotherapy response may overcome chemotherapy-induced growth and resistance, which is the major contributor to cancer-associated deaths.

The bone is the most common metastatic site for breast cancer, and is associated with poor prognosis and a 5-year survival rate of 15%. Moreover, patients exhibit substantial morbidity that profoundly impairs quality of life. While palliative treatments such as bisphosphonates are available to improve such symptoms and slow progression, bone metastases are resistant to anti-tumour treatments and therefore there remains no cure. With approximately 300,000 people living with bone metastasis in the US alone and an excess of 500,000 breast cancer-associated deaths worldwide annually, as well as an increasing incidence of breast cancer, strategies to prevent bone metastasis and reduce the associated morbidity remain to be of clinical importance.

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Appendices

Appendix 1: Materials list

Antibodies for flow cyometry			
Marker	Colour	Company	Clone
MSC panel			
Sca1	APC	BioLegend	D7
CD31	PE	BioLegend	WM59
CD45	APC Cy7	BioLegend	30-F11
CD45	PerCP/Cy5.5	BioLegend	30-F11
CD44	APC Cy7	BioLegend	IM7
CD11b	AF488	BioLegend	M1/70
CD29	APC	BioLegend	TS2/16
CD29	PE	eBioscience	HMb1-1
CD105	PerCPCy5.5	BioLegend	43A3
CD105	PE	eBioscience	MJ/18
CD34	BV605	BioLegend	581
Lymphoid panel			
B220	FITC	BioLegend	RA3-6BR
CD4	PerCPCy5.5	BioLegend	GK1.5
FOXP3	PE	ebioscience	FJK-16s
CD19	PE-Dazzle	BioLegend	6D5
CD3	PE Cy7	BioLegend	17A2
CD25	APC	BioLegend	PC61
CD45	APC Cy7	BioLegend	30-F11
NK1.1	BV650	BioLegend	PK136
CD8	AF700	BioLegend	53-6.7
Myeloid panel			
CD169	FITC	BioLegend	3D6.112
IL4R	PerCP Cy5.5	BD biosciences	11B11
CD204	PE	R&D	268318
F4/80	PE-Dazzle	BioLegend	BM8
Ly6G	PE Cy7	BioLegend	1A8
CCR2	APC	R&D	475301
CD45	APC Cy7	BioLegend	30-F11
Ly6C	BV510	BioLegend	HK1.4
CD115	BV 605	BioLegend	AFS98
CD11b	BV711	BioLegend	M1/70
Macrophage polarisation			
IL4R	PerCP Cy5.5	BD biosciences	11B11
CD204	PE	R&D	268318
PD1	PE-Dazzle	BioLegend	29F.1A12
PDL1	PE Cy7	eBioscience	MIH5
Tim4	AF647	BioLegend	RMT4-54
MHCII	AF700	BioLegend	M5/114.15.2
F4/80	BV650	BioLegend	BM8
CD45	APC Cy7	BioLegend	30-F11

Marker				Colour	Company	Clone
FACS						
Sca1	APC	BIOLEGEND	D7			
CD31	PE	BIOLEGEND	WM59			
CD45	APC Cy7	BIOLEGEND	30-F11			
CD44	APC Cy7	BIOLEGEND	IM7			
CD11b		BIOLEGEND	M1/70			
CD90		Biolegend	53-2.1			
CD29	APC	BIOLEGEND	TS2/16			
CD105	PerCPCy5.5	BIOLEGEND	43A3			
Ly6C	BV510	BIOLEGEND	HK1.4			
Ly6G						
CD34	BV605	BIOLEGEND	581			
Lymphoid panel						
B220	FITC	BIOLEGEND	RA3-6BR			
CD4	PerCPCy5.5	BIOLEGEND	GK1.5			
FOXP3	PE	ebioscience	FJK-16s			
CD19	PE-Dazzle	BIOLEGEND	6D5			
CD3	PE Cy7	BIOLEGEND	17A2			
CD25	APC	BIOLEGEND	PC61			
CD45	APC Cy7	BIOLEGEND	30-F11			
NK1.1	BV650	BIOLEGEND	PK136			
CD8	AF700	BIOLEGEND	53-6.7			
Myeloid panel						
CD169	FITC	BIOLEGEND	3D6.112			
	PerCP	BD				
IL4R	Cy5.5	biosciences	11B11			
CD204	PE	R&D	268318			
F4/80	PE-Dazzle	BIOLEGEND	BM8			
Ly6G	PE Cy7	BIOLEGEND	1A8			
CCR2	APC	R&D	475301			
CD45	APC Cy7	BIOLEGEND	30-F11			
Ly6C	BV510	BIOLEGEND	HK1.4			
CD115	BV 605	BIOLEGEND	AFS98			
CD11b	BV711	BIOLEGEND	M1/70			
Mac polarisation						
	PerCP	BD				
IL4R	Cy5.5	biosciences	11B11			
CD204	PE	R&D	268318			
PD1	PE-Dazzle	BIOLEGEND	29F.1A12			
		E-				
PDL1	PE Cy7	BIOSCIENCE	MIH5			
Tim4	AF647	BIOLEGEND	RMT4-54			
MHCII	AF700	BIOLEGEND	M5/114.15.2			
F4/80	BV650	BIOLEGEND	BM8			
CD45	APC Cy7	BIOLEGEND	30-F11			

Materials		
Item	Company	Cat no.
5'-azacitidine	Sigma	A2385-100mg
Alizarin red s	Sigma	A5533-25g
Amem	Gibco	22571-020
Ammonium	Merck	1054321000
Anti-biotin microbeads	Miltenyi Biotec	130-090-485
Benzyl alcohol	Sigma	305197-100ml
Benzyl benzoate	Sigma	136630-500ml
B-glycerophosphate	Sigma	G5422-25g
Bovine serum albumin	Sigma	A1470-100G
Citrate buffer	Sigma	C9999-1000ml
Collagenase, type II, powder	Life Technologies	17101-015
Compensation beads	eBioScience/Thermo	01-2222-42
Control liposomes	Liposoma	P-005
Corn oil	Sigma	C8267-500ml
Crystal violet	ACS (powder)	C6158-50g
Dexamethasone	Sigma	D4902-25mg
Diphtheria toxin	Sigma	D0564-1mg
D-luciferin (potassium salt)	Caymen Chemical Company	14681
DMEM	Gibco	41966-029
DMSO	VWR	0231-500ml
DNAeasy isolation kit	Qiagen	69504
Dnase I	Roche	10104159001
Doxorubicin	LKT laboratories	D5794-50mg
DPBS, no calcium, no magnesium (PBS)	Gibco	14190-014
Dual-luciferase® reporter assay system	Promega	E1910
Eosin	Thermo	6766007
Ethanol absolute 99.8+%,	Fisher	10437341
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma	E5134SLBZ3969
Foetal bovine serum	Life Technologies	10500064
Formaldehyde solution	Sigma	58775-500ml
Glu-52 Diphtheria toxin	Sigma	D2189-500ug
Glutamax™ Supplement	Thermo	35050038
Haematoxylin	Sigma	GH5316-500ml
HBSS, calcium, magnesium, no phenol red	Life Technologies	14025-092
Heparin sodium salt from porcine intestinal mucosa	Sigma	H4784
Histoclear	National Diagnostics	5989-27-5
Hygromycin	Invitrogen	10687010
Insert, 24 Well, PET 8µm, TL (Transwells 8.0um)	Sarstedt	83-3932-800
Insert, 6 Well, PET 0.4µm, TL (Transwell 0.4um)	Sarstedt	83-3432-04

Role of the tumour microenvironment in breast cancer bone metastasis

iSpacer®	SunJinLab	15013
L-ascorbic acid	sigma	A4544-100g
LB agar	Invitrogen	22700-025
L-glutamine	Thermo	25030081
Liberase DL	Roche	5466202001
Liberase TL	Roche	5401020001
Liposome clodronate	Liposoma	C-005
LS columns	Miltenyi Biotec	130-042-401
Mix and Go Competent cells	Cambridge (Zymo)	T3020
Normal goat serum	abcam	ab7481
Nuclease Free Water (not DEPC-Treated)	Ambion	AM9937
Oil red O	sigma	O0625-25g
Paclitaxel	LKT laboratories	D5794-50mg
Paraformaldehyde (PFA)	Sigma	P6148
Penicillin-Streptomycin (10,000 U/ml)	Gibco	15140-122
Pertex	SLS Ltd	3808707E
Plasmocin™ prophylactic	Invitrogen	Ant-mpt
Plasmocin™ treatment	Invitrogen	Ant-mpt-1
Polybrene	Santa-Cruz	sc134220
Poly-d-lysine	R&D systems	3439-200-01
Poly-ethyl glycol (PEG)	Sigma Aldrich	409528-500ml
Qtracker 705 Cell Labelling kit (qdot)	Thermo	Q25069
Quadrol (N,N,N',N'-Tetrakis(2-hydroxypropyl)ethylenediamine)	Aldrich	122262-1l
Rapiclear 1.52	SunJinLab	RC15200
RBC lysis buffer (10x)	BioLegend	420301
Shandon™ Cryomatrix™ embedding resin (OCT)	Thermo	6769006
Simplex Rapid Powder (dental cement)	Kemdent	ACR804
SOC media	LifeTechnologies	15544034
Sodium pyruvate	Gibco	11360039
Sterilin™ white microtiter™ plates	Thermo Fisher	611F96WT
Sucrose	SLS Ltd	CHE3650
Sudan Black B, high purity biological stain	VWR International Ltd	J6226822
Tamoxifen	Sigma	T5648-1g
Tert-Butanol	Honeywell/Sigma	360538-500ml
Thiazolyl blue tetrazolium bromide (MTT)	Sigma	M5655-100mg
Triethanolamine (TEA)	Sigma	90279-500ml
Tris Buffered Saline	Corning	46-012-CM
Triton x-100	Sigma	T8787-50ml
Trypsin-EDTA 0.25%	Gibco	25200-056
Vectashield	Vector Laboratories	H-1400
Vybrant DiD cell labelling solution	Thermo	V22887

Role of the tumour microenvironment in breast cancer bone metastasis

Zyppy Plasmid Miniprep kit	Zymo	D4020
Luciferase assay	Promega	E1500
Vetbond tm	3M	1469

Appendix 2: Publications, conferences and symposiums

Graham, N. & Qian, B. Z. Mesenchymal stromal cells: Emerging roles in bone metastasis. *Int. J. Mol. Sci.* **19**, (2018).

Intra-vital and advanced optical imaging symposium, 10th February 2016, Edinburgh

3rd Annual Symposium: Metchnikoff's legacy – 100 years of macrophages, 7th November 2016, Edinburgh

3Rs Symposium, 27th May 2016, Edinburgh

Light Sheet Microscopy Meeting, 27th March 2018, Edinburgh

Macrophage Satellite Symposium, Macrophages@EdUNI, 1st May 2018

A cell for all seasons: Macrophages in Health and Disease, 4th Annual Symposium, 2nd May 2018, Edinburgh

Edinburgh-Glasgow Cancer Immunology Meeting, 12th October 2018, Edinburgh

Edinburgh Super-Resolution Imaging Consortium (ESRIC) symposium, 28th May 2019, Edinburgh

Primary Science Teaching Trust Children's Conference, 6-8th June 2019, Edinburgh

A cell for all seasons: the 5th Annual Macrophages Symposium, 23rd October -24th October 2019, Edinburgh

Edinburgh Breast Cancer Special Symposium, 29th February 2020, Edinburgh

American Association for Cancer Research, Virtual Annual Meeting, 27-28th April 2020

Abstract and poster Submitted for: Edinburgh Breast Cancer Special Symposium, Edinburgh, UK, 29th February 2020

The role of mesenchymal stem cells in breast cancer bone metastasis

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Background:

The bone is the most prevalent metastasis site for breast cancer with 70% of patients with advanced disease displaying bone metastasis. Patients not only experience substantial morbidity such as pain and increased risk of fracture, but also exhibit a poor prognosis with a 5-year survival rate of 26%. Whilst palliative treatments to alleviate symptoms are available, these do not significantly enhance survival and there currently remains no cure.

The importance of the tumour microenvironment (TME) in cancer development is now evident. By establishing interactions between the tumour cells and components of the TME, such as immune cells and mesenchymal stromal cells, the TME plays a role in growth, metastasis and therapy resistance. An abundant component of the TME is mesenchymal stem cells (MSCs), which are multipotent cells that are involved in tissue maintenance and repair during wound healing. Within healthy bone, MSCs are a rare population making up approximately 0.001-0.01% of total cells and are defined by Nestin and CD146. They contribute to both bone homeostasis as well as the perivascular and endosteal haematopoietic stem cell niche. MSCs are recruited to tumours where they have been demonstrated to promote tumour development, metastasis and resistance. However, research into the role of MSCs in bone metastasis is limited, and will therefore be investigated in a model of breast cancer bone metastasis.

Methods

Experimental breast cancer bone metastases were induced via the intrailiac injection of MetBo2 cells, a bone metastatic subclone of breast cancer cells derived from the PyMT mouse. To investigate the role of MSCs in bone metastatic growth, bone metastasis were formed in Nestin-GFP-CreER^{T2}-iDTR mice, which permit the conditional depletion of Nestin⁺ MSCs. After tumour detection, MSCs were depleted and the effect on tumour growth measured using bioluminescent imaging.

Immunofluorescent staining of MSC markers, Nestin-GFP and CD146, was performed on bone samples and imaged using confocal microscopy to analyse the distribution of MSCs within normal and depleted bone metastasis.

In vivo experiments were complemented with *in vitro* assays including luciferase and transwell assays to measure the effect of MSCs on tumour cell proliferation.

Results:

Immunofluorescent staining of CD146 and Nestin-GFP demonstrates an increased infiltration of MSCs in bone metastasis in comparison to normal bone where MSCs are limited to the perivascular location.

Bone metastases in non-depleted mice exhibit increased growth over time. In contrast, MSC depletion induced during early stage bone metastasis results in a significant decrease in bone metastatic growth with tumours becoming 1/3 of their size before depletion and over 60x smaller than non-depleted controls.

In vitro proliferation assays demonstrated that direct co-culture of MetBo2 cells and bone marrow-derived MSCs results in a MSC number-dependent increase in tumour cell growth. Conversely, co-culture of MSCs with MetBo2 using a 0.4µm transwell had no significant effect on tumour cell proliferation.

Discussion/Conclusions:

Here, we present the evidence that Nestin⁺ MSCs play a significant role in the growth of breast cancer bone metastasis. Bone metastasis are infiltrated with MSCs, which when depleted using Nestin-GFP-CreER^{T2} -iDTR mice results in a near elimination of bone metastasis *in vivo*.

The molecular mechanism responsible for the effects of MSCs on tumour growth remains to be elucidated. Co-culture assays indicate that the molecular mechanism is dependent on direct contact between MSCs and tumour cells, as the transwell system, which only permits exchange of soluble factors, has little effect on tumour growth.

RNA-sequencing will be performed on Nestin-GFP⁺ MSCs and tumour cells to identify potential molecular interactions responsible for the bone metastatic growth. Future experiments will utilise molecular knockouts *in vivo* to test potential mechanisms functionally. This may offer insight into novel therapeutic targets to treat bone metastasis.

References:

Graham N & Qian B-Z. Mesenchymal Stromal Cells: Emerging Roles in Bone Metastasis. *Int J Mol Sci* 2018; 19(4):121.

Méndez-Ferrer S *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2011; 466(7308): 829-834.

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