Development of a Haematopoietic Stem Cell-based Cell Therapy to Treat Brain Metastases

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Abstract

Over the last decades, the occurrence of patients with brain metastases, originating mostly from melanoma, lung and breast cancer, has increased. Despite some progress, there are still no effective therapies that target brain metastases. Due to the blood-brain barrier, which restricts the access of conventional therapies to the central nervous system, therapeutic strategies need to include novel means of drug delivery. Furthermore, these therapies have to target multiple lesions simultaneously, as brain lesions often present multifocally.

This study aimed to develop a Haematopoietic stem cell (HSC)-based therapy that has the potential to overcome these limitations. In doing so, the suitability of HSCs and their myeloid progeny as cellular delivery vehicles for the delivery of genetically encoded therapeutic molecules into brain metastases was investigated. A strong infiltration of murine and human brain metastases tissue by the myeloid progeny of HSCs, which mostly consisted of macrophages, was demonstrated. Moreover, following *ex vivo* modification, the progeny of HSCs were able to deliver an expressed transgene to the proximity of brain metastases in preclinical models. To reduce the toxic effect of the delivered therapeutic molecules, an enzyme prodrug approach was developed and tested in the context of HSC therapy for targeting of brain metastases.

In addition to homing to brain metastases, the progeny of HSCs also infiltrates organs. In the context of the cell therapy this could lead to the accumulation of therapeutic molecules at those sites, resulting in possible side effects of the therapy. To address this issue, three promoters with high specificity and activity in murine and human brain metastases-infiltrating myeloid cells were identified, which could be used to restrict the delivery of genetically encoded therapeutic agents to brain metastases.

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5-FC	5-Fluorocytosine
5-FdUMP	5-Fluoro-2’-deoxyuridine-5’-monophosphate
5-FU	5-Fluorouracil
5-FUDP	5-Fluorouridine-diphosphate
5-FUTP	5-Fluorouridine-triphosphate
AD	Alzheimer’s disease
ADA	Adenosine deaminase
ADEPT	Antibody-directed enzyme prodrug therapy
ALD	Adrenoleukodystrophy
ALS	Amyotrophic lateral sclerosis
A-MuLV	Abelson leukaemia virus
AP-1	Activator protein 1
APC	Allophycocyanin
Arg1	Arginase 1
Art	Artemis
ATCC	American Type Culture Collection
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BCBM	Breast cancer brain metastases
bCD	Bacterial cytosine deaminase
bm	Bone marrow
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CD	Cytosine deaminase
cDNA	Copy DNA
CE	Carboxyl esterase
CFS	Cerebrospinal fluid space
CGD	Chronic granulomatous disease
CIAP	Calf intestine alkaline phosphatase
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CNS	Central nervous system
COX2	Cyclooxygenase 2
CPT-11	Irinotecan
CRL	Charles River Laboratories
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocytes
CMV	Cytomegalovirus
Dab2	Disabled homologue 2
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DEAE	Diethylaminoethylcellulose
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate

Dox	Doxycycline
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Egr-1	Early growth response 1
EP	Erythrocyte progenitor
ER	Oestrogen receptor
ERT	Enzyme replacement therapy
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FD	Fast digest
Flk	Foetal liver kinase
FLT-3	Fms-related tyrosine kinase 3 ligand
Fluc	Firefly Luciferase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GCV	Ganciclovir
GDEPT	Gene-directed enzyme prodrug therapy
gDNA	Genomic DNA
GDNF	Glial cell line-derived neurotrophic factor
GEX	Gene expression profiling
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GMP	Granulocyte/ macrophage progenitors
GP	Granulocyte progenitor
GR	Glucocorticoid-responsive element
GvHD	Graft-versus-host disease
HBEGF	Heparin-binding EGF-like grwoth factor
HD	Huntington’s disease
HEB	Basic helix-loop-helix protein
HEK	Human embryonic kidney
HER2	Human epidermal growth factor receptor 2
hESC	Human embryonic stem cells
HGF	Hepatocyte growth factor
HGF/SF	Hepatocyte growth factor/scatter factor
HGNC	HUGO gene nomenclature committee
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-DR	Class II major histocompatibility complex surface receptor
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
Hsp70	Heat-shock protein 70
HSV TK	Herpes simplex virus thymidine kinase
IF	Immunofluorescence
IFN	Interferon
IGF-1	Type 1 insulin growth factor

IL	Interleukin
ip	Intraperitoneal
iPS	Induced pluripotent stem cells
iv	Intravenous
LB	Lysogeny broth
Lin	Lineage antigens
LPS	Lipopolysaccharide
LRP-1	Lipoprotein receptor-related protein-1
LT	Lymphotoxin
LTR	Long terminal repeats
MacP	Macrophage progenitor
MACS	Magnetic activated cell sorting
MCP	Macrophage chemoattractant protein
MDSC	Myeloid-derived suppressor cells
MEP	Megakaryocyte/erythrocyte progenitors
miRNA	MicroRNA
miRT	MiRNA-targeting elements
MkP	Megakaryocyte progenitor
MLD	Metachromatic leukodystrophy
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
MOI	Multiplicity of infection
MPP	Multipotent progenitors
MRP	Multi-drug resistance protein
MSC	Mesenchymal stem cell
NBF	Neutral buffered formalin
NC	Negative control
NF-I	Nuclear factor-I
NK	Natural killer
NSC	Neural stem cell
OCT	Optimal cutting temperature compound
OCT1	Octamer transcription factor 1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PFA	Paraformaldehyde
PgP	P-glycoprotein
PPT	Polypurine tract
PR	Progesterone receptor
qPCR	Quantitative PCR
RAE	Ras-activated enhancer
RIPA	Radioimmunoprecipitation assay
Rlx	Relaxin
RMT	Receptor-mediated transcytosis
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute

RS-SCID	Radiosensitive severe combined immunodeficiency
RT	Reverse transcriptase
rtTA	Tetracycline-responsive transactivator
sCD	secretable CD
sCDHA	HA-tagged sCD
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SIN	Self inactivating
SN-38	7-ethyl-10-hydroxycamptothecin
Sp1	Specificity protein 1
Spp1	Osteopontin
SRF	Serum response factor
SRS	stereotactic radiosurgery
ssRNA	Single stranded ribonucleic acid
ST6GALNAC5	α-2,6-sialyltransferase
STAT3	Signal transducer and activator of transcription 3
T-ALL	T cell acute lymphocytic leukaemia
TAM	Tumour-associated macrophages
TAS	Transcriptional amplification strategy
TEM	Tie2-expressing monocytes
Tf	Transferrin
TfR	Transferrin receptor
TGF-β	Transforming growth factor β
Timp1	Tissue inhibitor of metalloproteinase 1
TNBC	Triple-negative breast cancer
TNF	Tumour necrosis factor
TNFR1	TNF receptor 1
tPA	Tissue plasminogen activator
TPO	Thrombopoietin
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRE	Tetracycline-responsive element
Treg	Regulatory T cells
TSS	Transcription start sites
TβRIIDN	Dominant-negative mutant of TGF-β receptor II
UbC	Ubiquitin C
UbC-GFP	Expression of GFP controlled by human UbC promoter
UPRT	Uracil phosphoribosyltransferase
UTR	Untranslated region
VDEPT	Virus-directed enzyme prodrug therapy
VEGF	Vascular endothelial growth factor
VLCFA	Very long-chain fatty acid
VSV-G	Vesicular stomatitis virus-glycoprotein
WAS	Wiskott-Aldridge syndrome
WBC	White blood cell
WBRT	Whole brain radiation therapy

X-SCID	X-linked severe combined immunodeficiency
yCD	Yeast cytosine deaminase

1. Introduction

1.1 Brain metastases

Brain metastases are the most frequently occurring brain tumours in adults with an annual incidence in the USA and Europe in the range of 5-10 per 100,000 per year (Stark, 2011). An estimated 20 to 35% of all cancer patients develop brain metastases (Gavrilovic and Posner, 2005) with most originating from primary cancers in the lung (40–50%), breast (15–25%), or from melanoma (5–20%) (Schouten et al., 2002, Barnholtz-Sloan et al., 2004). The frequency of diagnosis of metastatic brain tumours is increasing (Eichler et al., 2011) due to a combination of improved imaging approaches, earlier detection, and the longer survival of patients after primary cancer diagnosis.

Central nervous system (CNS) metastases are a major cause of morbidity and mortality in patients with solid tumours affecting survival, neurocognition, speech, coordination, behaviour, and quality of life (Mehta et al., 2003, Chang et al., 2007). These clinical features might be as a result of destruction or displacement of brain tissue by the expanding tumour, peritumoural oedema leading to further disruption of surrounding white matter tracts, increased intracranial pressure, and/or vascular compromise (Eichler et al., 2011). Furthermore, the majority of patients exhibit multiple tumours at the time of diagnosis.

1.1.1 Breast cancer brain metastases

An estimated 10% to 30% of all breast cancer patients will eventually develop brain metastases (Lin et al., 2004). These can develop in sites in the CNS, such as the brain, cranial nerves, spinal cord, leptomeninges, and eyes. About 2 to 5% of breast cancer patients develop leptomeningeal metastases (DeAngelis et al., 2000), whereas the incidence of epidural spinal cord metastases in patients with breast cancer is reported to be 4% (Hill et al., 1993). Furthermore, breast cancer can metastasise to the eye at a higher rate than other primary cancers (McCormick and Abramson, 2000).

Compared with other sites of metastatic spread, the diagnosis of breast cancer brain metastases (BCBMs) is associated with the shortest survival time (Kennecke et al., 2010). The outcome in patients with BCBMs strongly correlates with the tumour subtype and performance status of the patient (Sperduto et al., 2012, Berghoff et al., 2012). The incidence of brain metastasis in patients with early-stage breast cancer are highest in those with human epidermal growth factor receptor 2-positive ($HER2^+$ or ERBB2) and triple-negative breast cancer (TNBC: oestrogen receptor-negative (ER^-), progesterone

receptor-negative (PR^-), and HER2-negative ($HER2^-$) disease) and lowest in ER-positive (ER^+) disease (Kennecke et al., 2010).

The median time interval from primary diagnosis to the development of BCBMs is the shortest in TNBC (27.5 months) and $HER2^+$ disease (35.8 months), and longer in patients with $ER^+/HER2^-$ (54.4 months), and $ER^+/HER2^+$ disease (47.4 months). These trends also apply to the median survival following the diagnosis of BCBMs with 7.3 months for TNBC, 17.9 months for $HER2^+$ disease, 10 months for $ER^+/HER2^-$ and 22.9 months for $ER^+/HER2^+$ disease, as reported by a large retrospective study of 865 patients with BCBMs (Sperduto et al., 2013). The development of new therapeutic strategies for BCBMs, particularly in the TNBC and $HER2^+$ breast cancer subtypes is therefore of great interest.

With the advances in HER2-directed therapies and their routine use to treat $HER2^+$ breast cancer patients, the pattern of breast cancer dissemination in this patient group has changed. In patients that have received adjuvant HER2-directed systemic therapies, relapse in the CNS is rare (2% with trastuzumab and 1% with lapatinib) (Pestalozzi et al., 2013, Goss et al., 2013). However, approximately 30% to 55% of patients with metastatic $HER2^+$ disease will eventually develop brain metastases and up to 50% of these patients will die from progressive CNS disease (Bendell et al., 2003, Kennecke et al., 2010, Brufsky et al., 2011, Olson et al., 2013). Patients with TNBC have a high risk of CNS relapse (25% to 46%) (Lin et al., 2008, Kennecke et al., 2010, Lee et al., 2011). However, extracranial disease progression in TNBC patients is common and this mostly occurs in the early phase of the disease course (Lin et al., 2008, Dawood et al., 2008, Dawood et al., 2012) and as a result TNBC patients with BCBMs rarely die from progressive CNS disease alone (Lin et al., 2008). There is, therefore, an urgent need to develop additional systemic therapies that are effective in controlling intra- and extra-CNS disease concurrently for TBNC patients.

Current treatment options for breast cancer brain metastases are limited, and there are still no systemic regimens that have been developed for this setting. Patients with BCBMs were once routinely excluded from clinical trials, whereas now an increasing number of trials investigating novel systemic approaches specific to BCBMs are available to them (Lin, 2013). However, much progress remains to be made.

1.1.2 Brain metastasis formation

Metastasis formation is a cascading process whereby cancer cells escape from the primary tumour site, invade surrounding tissue, intravasate into the bloodstream or lymphatics, and arrest, extravasate, survive and proliferate within a secondary site. Certain tumour types demonstrate an organ‑specific pattern of dissemination. The propensity of cancer cells to spread to specific sites was hypothesised to be influenced by the interaction between a tumour cell (the 'seed') and a congenial microenvironment (the 'soil') by Paget (1989). However, Ewing proposed that circulatory patterns between the primary tumour and specific secondary organs are sufficient to explain the majority of organ-specific metastatic spread (Ewing, 1928). Recently, it has been demonstrated that cancer cells can bring their own soil, e.g. stromal components from the primary site including activated fibroblasts, to secondary sites (Duda et al., 2010). There are at least three brain microenvironments implicated in metastatic colonisation: the perivascular niche, the brain parenchyma and the cerebrospinal fluid (CSF) or the leptomeningeal niche (Steeg et al., 2011).

Brain metastases most frequently derive from lung (40–50%), breast cancer (15–25%), or melanoma (5–20%) (Schouten et al., 2002, Barnholtz-Sloan et al., 2004). Metastatic cancer cells shed from the primary cancer and invade the surrounding tissue until they intravasate into the bloodstream or lymphatics (Figure 1-1). These cells can enter the brain circulation, where they may arrest in sites of slow flow within the capillary bed at vascular branch points (Kienast et al., 2010). This is followed by early changes in the brain microenvironment (Lorger and Felding-Habermann, 2010). The arrested tumour cells elongate their shape along brain vascular endothelial cells. They adhere to the vascular basement membrane via β1 integrins, and proliferate and invade while on top of the vascular basement membrane (Carbonell et al., 2009). To extravasate across the blood-brain barrier (BBB), cancer cells first cross the endothelial cell layer to enter the perivascular space between endothelial cells and their supporting astrocytic end-feet. There, tumour cells can reside for a while and may only cross the astrocytic end-feet after expansion to enter the brain parenchyma. The cancer cell trans-BBB migration is mediated by various proteins, including heparin-binding epidermal growth factor (EGF)-like growth factor (HBEGF), cyclooxygenase 2 (COX2), and α-2,6-sialyltransferase (ST6GALNAC5) (Bos et al., 2009). After tumour cell extravasation, metastatic cells can either stay dormant or begin colonisation in the brain parenchyma. The latter process is partly supported by the brain tumour microenvironment and begins with the activation of survival and proliferation processes of the cancer cells.

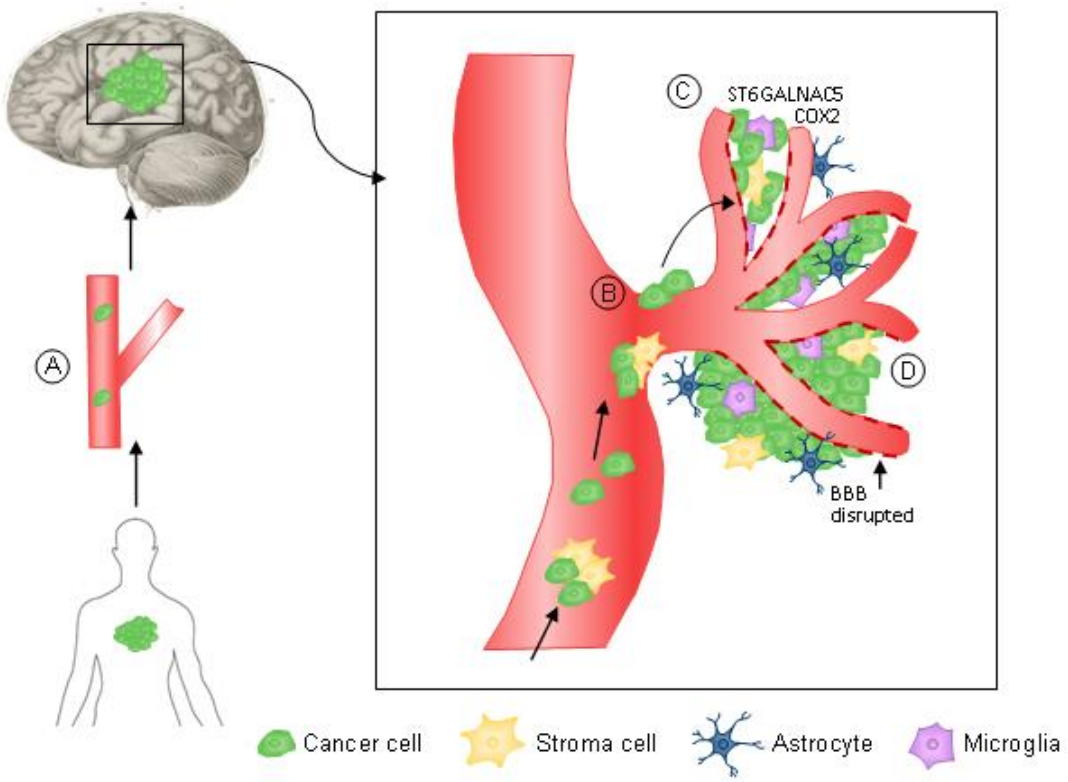


Figure 1-1: Steps in the formation of haematogenous metastasis to the brain (adapted from Eichler et al. (2011)). **(A)** Metastasis formation is a cascading process, whereby cancer cells escape from the primary tumour site (lung (40–50%), breast cancer (15–25%), or melanoma (5–20%)), invade surrounding tissue, and intravasate into the bloodstream or lymphatics. **(B)** Metastatic cancer cells can enter the brain circulation, where they arrest within the capillary bed at vascular branch points. **(C)** Following the extravasation across the BBB, the cells enter the brain parenchyma. The cancer cell trans-BBB migration is mediated by different proteins. **(D)** After extravasation the colonisation of the brain starts. Survival and proliferation of the tumour cells are partly supported by the brain tumour microenvironment and by the recruitment of new blood vessels.

1.1.2.1 Brain tumour microenvironment

The brain provides a tumour environment that differs from most other organs. Once cancer cells infiltrate brain tissue they can encounter a number of host cell types, including brain endothelial cells, cells of myeloid origin (such as microglia and macrophages), other cells of the immune system, astrocytes as well as co-disseminating stromal cells.

Myeloid cells

There are different cell populations of myeloid origin including microglia, tumour-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC) and Tie2-expressing monocytes (TEM). All these myeloid cells are characterised by the expression of cell surface marker CD11b, and have been shown to infiltrate malignant brain lesions and to play a role in brain tumour progression.

The CNS contains two different types of macrophages, including parenchymal microglia and perivascular macrophages (Davis et al., 1994, Guillemin and Brew, 2004). Perivascular macrophages are constitutively replenished by circulating monocytes (Hickey and Kimura, 1988), whereas brain-resident microglia have been shown to originate from the umbilical vesicle (Ginhoux et al., 2010). Several studies have shown that monocytes can infiltrate the adult brain and differentiate into parenchymal microglia, although the monocyte turnover is very slow (Davis et al., 1994, Guillemin and Brew, 2004, Hess et al., 2004, Soulas et al., 2009) and restricted to specific brain regions (Vallieres and Sawchenko, 2003). Moreover, infiltrating inflammatory monocytes have been shown to be the main source of microglia/macrophages under pathologic conditions such as viral encephalitis and Alzheimer's disease (Biffi et al., 2004, Getts et al., 2008).

Activated microglia and macrophages are frequently found to infiltrate primary and metastatic brain tumours in patients and experimental models (He et al., 2006, Daginakatte and Gutmann, 2007, Fitzgerald et al., 2008, Lorger and Felding-Habermann, 2010). However, the mechanism by which the recruitment of macrophages/microglia by brain metastases occurs is largely unknown. However, primary brain tumours such as gliomas have been shown to attract monocytes via the production of macrophage chemoattractant protein 1 and 3 (MCP-1, MCP-3) and hepatocyte growth factor/scatter factor (HGF/SF) (Strik et al., 2004, Okada et al., 2009).

The production of cytokines, growth factors, and enzymes by microglia/macrophages can directly or indirectly contribute to angiogenesis, tumour cell proliferation, and invasion of metastatic cancer cells to the brain (Davis et al., 1994, Guillemin and Brew, 2004, Markovic et al., 2005, Hoelzinger et al., 2007, Fitzgerald et al., 2008, Markovic et al., 2009). Several studies using experimental glioma models have demonstrated that myeloid cells contribute to tumour progression (Daginakatte and Gutmann, 2007, Markovic et al., 2009). By contrast, anti-tumour activity of myeloid cells in the brain has also been demonstrated (Kanamori et al., 2006, Galarneau et al., 2007). Moreover, microglia exhibited potential tumour cytotoxicity towards lung cancer brain metastases

in an *in vitro* study (Murata et al., 1997). In addition, *in vitro* co-cultures have demonstrated that activated microglia create an altered brain microenvironment that promotes tumour growth and invasion (Fitzgerald et al., 2008). Furthermore, it has been shown that microglia can enhance the invasion and colonisation of brain tissue by breast cancer cells (Joyce and Pollard, 2009, Pukrop et al., 2010).

TAMs can either be in a M1 (tumour-suppressive) or M2-like (tumour-supportive) state. Investigations have shown that TAMs mainly acquire M2-like properties in most cancers with potent immunosuppressive functions (Mantovani et al., 2002). Macrophages are activated by the T_h1-type cytokines interferon-γ and lipopolysaccharide (LPS), resulting in up-regulation of nitric oxide synthase 2 (Nos2) and in a pro-inflammatory M1 phenotype. The T_h2-type cytokines interleukin 4 and 13 induce the tumour-supportive M2-like state of TAMs, resulting in the up-regulation of arginase 1 (Arg1) (Mills et al., 2000, Mantovani and Sica, 2010). The presence of TAMs in many solid tumours is correlated with poor prognosis. Moreover, the development of metastases in mice deficient in macrophages was prevented, while the progression of the primary neoplasm was not hindered (Lin and Pollard, 2004). It has also been shown that macrophages are recruited to metastatic sites prior to metastatic cell recruitment, suggesting a role for macrophages in the preparation of the premetastatic niche (Mareel and Madani, 2006).

A subset of circulating and tumour-infiltrating monocytes expressing the angiopoietin receptor Tie2 are called Tie-2 expressing monocytes (TEM) (De Palma et al., 2005). TEMs have been shown to exhibit the highest proangiogenic activity of all tumour-infiltrating myeloid cells in glioma (De Palma et al., 2005). It has been shown that TEMs are derived from resident blood monocytes and are strongly polarised towards the M2-like state (Pucci et al., 2009). Therefore, it has been suggested that resident and inflammatory monocytes may be differentially committed to generate tumour infiltrating TEMs and TAMs, respectively (Pucci et al., 2009). The M2 phenotype of TEMs seems to be stronger than in TAMs with an enhanced proangiogenic and reduced pro-inflammatory activity.

MDSCs are a mixed myeloid cell population with immunosuppressive activity (Talmadge, 2007, Gabrilovich and Nagaraj, 2009, Greten et al., 2011). The numbers of MDSCs in human and murine blood increase dramatically under pathologic conditions and amass in pathologic lesions including tumours (Melani et al., 2003, Greten et al., 2011). Moreover, MDSCs have been shown to be a main source of the immunosuppressive molecule transforming growth factor β (TGF-β), and to inhibit T-cell activation in an experimental glioma model (Umemura et al., 2008).

In summary, different populations of myeloid-derived cells display diverse effects on intracranial tumour growth. However, these effects can also be influenced by the tumour type and its molecular background.

Other immune cells

Natural killer (NK) cells are an important part of the innate immune system, and are known to play a role in the apoptotic killing of tumour cells (Trinchieri and Perussia, 1984, Trinchieri, 1989, Yang et al., 2006). NK cell infiltration into extracranial tumours has been correlated with increased patient survival (Ishigami et al., 2000, Vujanovic, 2001). Brain metastatic lesions are frequently infiltrated by NK cells, however the proportion of these cells remain low (Vaquero et al., 1990). Experimental glioma studies have demonstrated a tumour-suppressing role for NK cells (Basse et al., 1988). Further, an anti-metastatic effect of NK cells has been observed in other organs (Yasumura et al., 1994, Dewan et al., 2005, Ksienzyk et al., 2011). However, the influence of NK cell depletion on the development of intracranial metastases has yet to be established.

Another population of lymphocytes are T lymphocytes that can be further classified into two categories: $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs). High levels of $CD4^+$ Th cells are positively correlated to tumour development, whereas high levels of $CD8^+$ CTLs are associated with tumour destruction (Gerloni and Zanetti, 2005). Moreover, a high ratio of CD8+ cells to CD4+ cells has been shown to be an indicator of less aggressive primary CNS tumours (Yu et al., 2003). It has been shown that the depletion of $CD4^+$ or $CD8^+$ T cell populations leads to an increase in brain metastases (Lu et al., 2003). Regulatory T cells (Treg) are important modulators of the immune system that have been shown to infiltrate experimental brain metastases and human metastatic brain lesions (Sugihara et al., 2009). Moreover, the presence of Tregs in tumours has been shown to correlate with poor clinical outcome (Curiel et al., 2004, Yu et al., 2005).

The role of B cells in the development of brain metastases is poorly understood. In experimentally induced melanoma metastases to the lung an increase in metastatic burden as a result of B cell depletion could be detected. Additionally, reduced activation of $CD4^+$ and $CD8^+$ T cells could be seen, suggesting a strong interaction between B cell and T cell populations (DiLillo et al., 2010). Further research on the B cell contribution to brain metastases has to be performed to elucidate this relationship.

All these cell populations may be used in future immunotherapy against metastatic brain tumours. However, further investigations into their contribution to metastasis formation in the CNS need to be performed.

Astrocytes

The star-shaped astrocytes usually support glial cells in providing protection and maintenance of the brain environment. Histological analysis of resected human brain metastases revealed a close association of tumour cells with activated astrocytes (Zhang and Olsson, 1997, Fitzgerald et al., 2008, Seike et al., 2011). The activation of astrocytes is also widely observed around experimental brain metastases (Fitzgerald et al., 2008, Lin et al., 2010, Seike et al., 2011).

Activated peritumoural astrocytes have been shown to express endothelin 1, a regulator of numerous transforming processes in 85% of metastases of the human brain (Zhang and Olsson, 1995). Additionally, tumour cells within brain metastases have been shown to produce factors, such as macrophage inhibitory factor, interleukin-8 (IL-8) and plasminogen activator inhibitor 1, that activate astrocytes which, in turn, produce proliferative factors for the tumour cells, such as IL-6, IL-1β and tumour necrosis factor (Seike et al., 2011). Furthermore, astrocytes have been shown to produce pro-matrix metalloproteinase 2 (pro-MMP2) and plasminogen activator and thereby promote glioma invasiveness (Le et al., 2003). Recently, it has been suggested that astrocytes may protect cancer cells from chemotherapy-induced apoptosis (Lin et al., 2010). Overall, astrocytes may contribute to cancer progression in the brain through a variety of different mechanisms.

1.1.2.2 Molecular pathways mediating brain metastases

To identify gene expression changes during metastasis to the brain, comparisons of matched and unmatched tissues blocks of primary tumour and brain metastases have been performed. These studies highlighted differences in the expression of stem cell markers, receptor tyrosine kinases, metastasis suppressor genes, hormone receptors, cyclooxygenase 2, proteins involved in apoptosis and DNA repair enzymes (Da Silva et al., 2010, Sun et al., 2009, Stark et al., 2005, Koo and Kim, 2011, Milas et al., 2003, Gaedcke et al., 2007, Wu et al., 2010, Gomez-Roca et al., 2009). All of these gene

expression changes represent potential leads for the functional modulation of brain metastatic formation.

To identify additional pathways that are involved in metastasis to the brain, gene expression changes between experimental brain-tropic and parental tumour cell lines have been performed. For example, the overexpression of ERBB2 in the brain variant of the MDA-MB-231 breast cancer cell line had no effect on the number of micrometastases, but increased the number of large metastases (Palmieri et al., 2007). Thus, ERBB2 overexpression is a key player in the final stages of metastatic colonisation in the brain, but did not promote the initial steps of tumour cell arrival or growth.

Overexpression of α-2,6-sialyltransferase (ST6GALNAC5) was identified in brain, but not in bone- or lung-tropic breast cancer cell lines. The inhibition of *ST6GALNAC5* reduced tumour cell line migration across artificial BBBs *in vitro* and enhanced brain metastasis-free survival in animal models (Bos et al., 2009).

Furthermore, the expression of proteases within the parenchyma as well as by the invading tumour cells has been shown to contribute to brain metastasis. A reduced incidence of brain metastasis in mice could be detected following overexpression of tissue inhibitor of metalloproteinase 1 (Timp1) and reduction of plasminogen activator inhibitor 1 expression (Kruger et al., 1998, Maillard et al., 2008). In addition, reduced heparanase expression in tumour cells led to decreased experimental brain metastasis (Zhang et al., 2011).

Other pathways essential for the colonisation of the brain by metastatic cells still need to be identified by future investigations. These pathways could be potential targets for future therapies.

1.1.3 Management of brain metastases

Despite the therapeutic options for brain metastases such as surgery, whole brain radiation therapy (WBRT), stereotactic radiosurgery (SRS), chemotherapy, growth factor inhibitors, or a combination of these therapies, outcomes remain poor (Eichler and Loeffler, 2007). The median survival time of patients with brain metastases is only 4 to 19 months (Ahn et al., 2012).

WBRT is the standard treatment of choice in cases of multiple brain metastases, in patients whose overall clinical condition is poor and rapidly deteriorating, and in patients for whom easy palliation is the goal. Survival for patients with brain metastases treated

with WBRT typically ranges from 4–6 months, but can be as long as 12–24 months for selected patients (Mehta et al., 2003). Strong positive prognostic factors include good functional status, age <65 years, no sites of metastases outside of the CNS, the presence of a single tumour lesion in the brain, long interval from primary diagnosis to brain relapse and a controlled primary tumour (Gaspar et al., 1997, Melisko et al., 2008). Further positive prognostic factors can also be certain cancer subtypes such as HER2-positive breast cancer and epidermal growth factor receptor (EGFR)-mutant non-small cell lung cancer (NSCLC) (Eichler et al., 2008, Eichler et al., 2010). The response can be predicted based on the radiosensitivity of the primary tumour.

Brain metastases patients with large single lesions that are immediately life threatening are treated with surgical resection. Randomised trials have shown a prolonged survival in patients that underwent resection plus WBRT compared with WBRT alone, median survival of 10 months versus 4-6 months, respectively (Patchell et al., 1990, Vecht et al., 1993). An improvement in neurologic functions and prolonged independence was also observed in patients receiving surgery plus WBRT, compared with WBRT alone.

SRS is a non-invasive alternative to surgery and is considered in patients with tumour diameters ≤3.5 cm. It also offers a treatment opportunity for patients with a single lesion that is not surgically accessible (e.g., in the brainstem) or for patients that are unable to undergo surgery for medical reasons. SRS is a highly focussed radiotherapy delivering multiple convergent beams specifically to the tumour, leaving the surrounding normal brain tissue intact. The beams can be accomplished by a linear accelerator, gamma knife or protons produced by cyclotron (Gerrard and Franks, 2004). The median survival of SRS treated patients can be extended to about 10 months (Nguyen and Deangelis, 2004). It has been shown in randomised clinical trials that surgery or SRS combined with WBRT improves overall survival compared with WBRT alone in patients with a single metastatic lesion in the brain (Patchell et al., 1990, Vecht et al., 1993). However, patients with four or fewer brain metastases treated with SRS demonstrated equivalent overall survival, but worse intracranial disease control compared with SRS plus WBRT (Andrews et al., 2004, Aoyama et al., 2006, Kocher et al., 2011).

Brain metastases are not routinely treated with chemotherapy. It is only used as salvage therapy in patients who failed to respond to WBRT, SRS and surgery. Response rates to varying regimens depend largely on the primary tumour sensitivity to chemotherapy (reviewed in Nguyen and Deangelis (2004)). Overall, chemotherapy clinical data for brain metastases are unambiguously disappointing, with only a few clinical responses to standard cytotoxic drugs (summarised in Steeg et al. (2011)).

All the above mentioned treatment options for brain metastases are only able to improve patients life for a limited time. Therefore, novel treatment strategies specifically targeting brain metastases need to be developed.

1.1.4 Novel strategies to improve drug delivery across the BBB

The main reasons for the poor efficacy of standard chemotherapeutic agents in brain metastases are the poor BBB penetrability of many systemically active chemotherapeutic drugs and the resistance to chemotherapeutics that may have occurred in patients that have had multiple rounds of chemotherapy prior to the development of CNS metastatic disease. More recently, new therapies and strategies are increasingly being explored in an effort to enhance drug delivery to the brain.

1.1.4.1 The blood-brain barrier (BBB)

In order to provide protection to brain cells as well as preservation of brain homeostasis, a specialised barrier system of endothelial cells is formed within the brain. This BBB separates the circulating blood from the brain extracellular fluid in the CNS. It occurs along all capillaries and is formed by specialised endothelial cells lining the cerebral microvasculature, together with pericytes and astrocytic perivascular endfeet (Figure 1-2). The endothelial cells of the BBB display tight junctions between adjacent cells and express an abundance of active transporter efflux pumps. Tight junctions create a physical barrier forcing molecules to pass through rather than between endothelial cells. Additionally, the efflux pumps send substances out of endothelial cells and back into the circulation, away from the brain parenchyma. Examples of these efflux pumps are the P-glycoprotein (PgP) or multi-drug resistance protein (MRP), which actively remove some chemotherapeutic drugs from the brain (Szakacs et al., 2006, Noguchi et al., 2009). Small and lipophilic molecules are not recognised by the active efflux pumps and can pass from blood into the brain (Pajouhesh and Lenz, 2005). Other substances, such as glucose, amino acids, vitamins, nucleic acid precursors and some hormones, are transported into the brain by facilitated diffusion (Ohtsuki and Terasaki, 2007). Large hydrophilic molecules, including many chemotherapeutic and molecular targeted drugs, are excluded from the CNS.

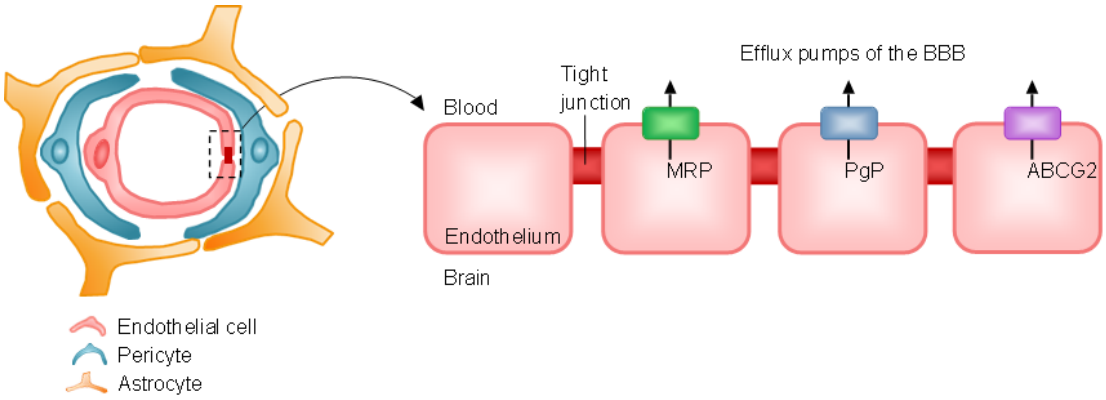


Figure 1-2: Schematic diagram of the BBB with an enhanced illustration of the brain capillary endothelial cell (adapted from Eichler et al. (2011)). The main drug efflux transporters of brain capillary endothelial cells include MRPs, PgP, and ABCG2.

The BBB becomes structurally and functionally compromised when brain metastases grow beyond 1-2 mm in diameter (Yuan et al., 1994, Hobbs et al., 1998, Monsky et al., 1999, Deeken and Loscher, 2007). It has been shown that lesions smaller than 0.2 mm^2 had an intact BBB in an experimental brain metastases model, whereas larger tumour-cell clusters resulted in leakage of sodium fluorescein (Zhang et al., 1992). Furthermore, the growing tumour mass led to the disruption of the interaction of astrocytes and endothelial cells. In addition to changes in blood vessel permeability, a significant reduction in endothelial PgP expression to 5% and 40% of normal levels in brain metastases from melanoma and lung carcinoma, respectively, could be detected (Regina et al., 2001). However, the disruption of the BBB is not homogeneous, and the BBB might remain intact in parts of tumours. Pharmacokinetic studies of two experimental brain metastases models revealed that most metastases have some increased permeability compared with normal brain. However, heterogeneous uptake levels can occur and only 10% of the experimental brain metastases had sufficient permeability to show a cytotoxic response to chemotherapy (Lockman et al., 2010). Since undiagnosed small brain metastases lesions in patients, capable of contributing to CNS recurrence, and some macroscopic tumours have a relatively intact BBB (Lockman et al., 2010), novel therapeutic approaches have to be able to overcome these barriers to deliver drugs to such lesions. This can be addressed in different ways, e.g. by physical and pharmacological disruption of the BBB, identification and development of brain metastases-permeable drugs, or the use of cell vehicles that can cross the BBB (Figure 1-3). The aim of this thesis was to develop an application of the latter strategy using HSCs as cellular vehicles. Other examples of this kind of approach are discussed separately in chapter 1.2.

1.1.4.2 Disruption of tight junctions

The penetration of water-soluble compounds across the BBB is restricted by the tight junctions present between endothelial cells. These tight junctions can be disrupted by mechanical and pharmacological methods. An intra-arterial infusion of a hyperosmotic agent injected prior to the administration of chemotherapeutics led to an osmotic disruption of the BBB, and therefore to an enhanced delivery of the chemotherapeutic in brain tumour patients (Bellavance et al., 2008). A BBB disruption can also be achieved by targeted ultrasound. To this end, preformed gas bubbles were intravenously injected in rat brain tumour models before a targeted pulsed ultrasound was applied, leading to BBB disruption and an increased uptake of chemotherapeutics into the brain parenchyma (Liu et al., 2010). Pharmacological disruption of the BBB can be achieved with bradykinin analogues such as RMP-7. Various studies have shown an increased uptake of standard chemotherapeutic agents in brain tumours following intravenous delivery of RMP-7 (Gregor et al., 1999, Matsukado et al., 1996, Prados et al., 2003). Additionally, preclinical studies have demonstrated the ability of recombinant human tumour necrosis factor (TNF), a proinflammatory cytokine, to disrupt endothelial tight junctions in the tumour vasculature through the RhoA/Rho kinase (Seki et al., 2011). The vascular endothelium associated with brain metastases showed localised expression of TNF receptor 1 (TNFR1) (Connell et al., 2013); and administration of TNF or its endogenous analogue lymphotoxin (LT) permeabilised the BBB to exogenous tracers selectively at sites of brain metastases.

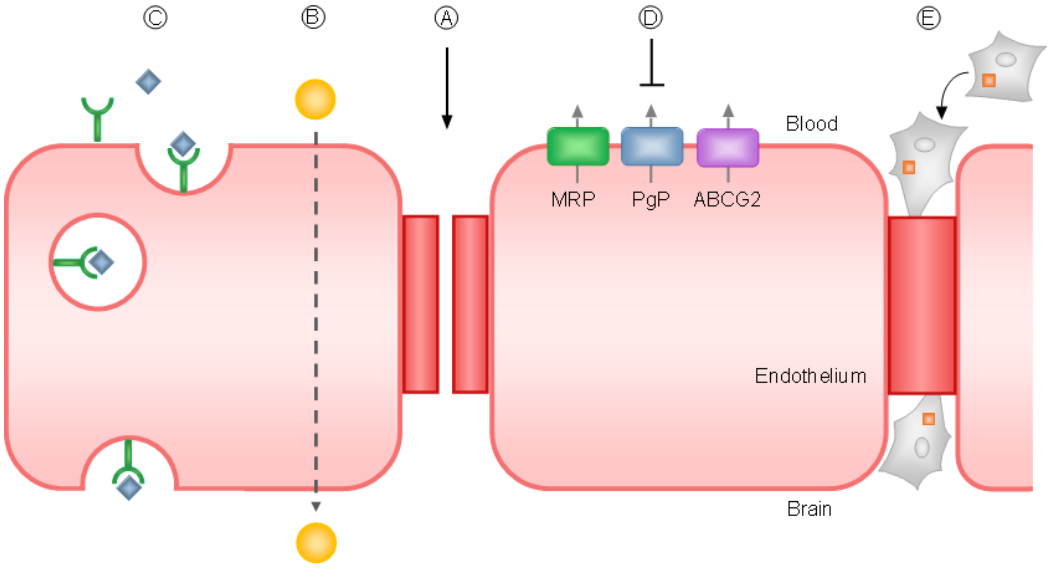


Figure 1-3: Modifications to improve brain drug delivery (adapted from Eichler et al. (2011)). **(A)** Tight junctions of endothelial cells of BBB can be disrupted by mechanical and pharmacological methods. **(B)** Brain metastases-permeable drugs that have demonstrated therapeutic efficacy. **(C)** Drugs can be delivered across the BBB by receptor-mediated transcytosis (RMT). **(D)** Inhibitors of main drug efflux transporters of brain capillary endothelial cells can facilitate delivery of brain metastases drugs across the BBB. **(E)** Cellular vehicles that can cross the BBB can be used to deliver drugs to the CNS.

1.1.4.3 Brain metastases-permeable drugs

Brain metastases-permeable drugs should have a low molecular mass (<450 Da), moderate lipophilicity (calculated logP<5), a limited number of hydrogen bond donors (less than three) and acceptors (less than seven), neutral or basic pK_a (7.5–10.5), and limited polar surface area (<60–79 Å) (Pajouhesh and Lenz, 2005). Several BBB-permeable drugs, such as vorinostat, lapatinib, WP1066 (signal transducer and activator of transcription 3 (STAT3) inhibitor), sagipilone, and pazopanib have already been tested in preclinical models as well as clinical trials. Vorinostat is a histone deacetylase inhibitor and when injected into mice with breast cancer brain metastases, it crossed the BBB, exhibited heterogeneous greater uptake in metastases, relative to normal brain, and led to reduced formation of large metastases and micrometastases (Palmieri et al., 2009). However, vorinostat monotherapy showed disappointing clinical activity against metastatic breast cancer (Luu et al., 2008), and in patients with advanced lung cancer. It displayed synergistic effects with carboplatinum and paclitaxel leading to increased response rates with a trend towards improved progression-free survival (Ramalingam et al., 2010). Further, the combination of vorinostat and radiation resulted in prolonged survival in breast cancer brain metastases models in mice (Baschnagel et al., 2009). Another BBB-permeable drug is lapatinib, an ERBB2 and EGFR kinase inhibitor. It has

been shown to cross the BBB and prevent the formation of metastases by brain-tropic breast cancer cells following systemic administration in tumour-bearing mice (Gril et al., 2008). A brain-permeable STAT3 inhibitor, WP1066, has also been shown to increase the overall survival in mice with melanoma brain metastases (Kong et al., 2008). It could also be shown that WP1066 reduced tumour cell production of TGFβ, vascular endothelial growth factor (VEGF) and other chemokines, as well as inhibiting the proliferation of regulatory T (Treg) cells and increasing cytotoxic T cell responses. Sagipilone is a BBB-permeable microtubule-active drug with a long half-life in the brain. It can inhibit the intracerebral growth of breast cancer and lung cancer cells compared with the effects of paclitaxel or temozolomide (Hoffmann et al., 2009).

1.1.4.4 Receptor-mediated transcytosis (RMT)

Receptor-mediated transcytosis (RMT) is another approach that can be used to bypass the BBB for the treatment of brain metastases. This involves the design of drugs that can be shuttled across the BBB using receptors that are naturally expressed on the brain endothelial cells. One receptor that is highly expressed at the BBB and involved in the transport of proteins and peptides is lipoprotein receptor-related protein (LRP-1) (Lillis et al., 2008). The peptide angiopep2 binds LRP-1 as a ligand, resulting in facilitated transport across the BBB. The drug GRN1005, which is paclitaxel linked to angiopep2, demonstrated an increased uptake into the brain and brain metastases compared with paclitaxel in the MDA-MB-231-BR mouse model (Thomas et al., 2009). Furthermore, GRN1005 showed anti-tumour activity in preclinical models, as well as in phase I clinical trials (Regina et al., 2008, Kurzrock et al., 2012, Drappatz et al., 2013). The transferrin receptor (TfR) is highly expressed by brain capillaries and mediates the delivery of iron to the brain (Jefferies et al., 1984). The natural ligand is the iron binding protein, transferrin (Tf) (Pardridge et al., 1987). Antibody/drugs fused with Tf have shown increased uptake into the CNS (Shin et al., 1995, Mishra et al., 2006). However, TfR is nearly saturated with the endogenous Tf in the bloodstream, indicating that a Tf-targeted drug would have to compete with the natural ligand (Qian et al., 2002). Bispecific antibodies with an epitope for TfR, to increase the CNS uptake of the antibody, and an epitope to target disease-specific proteins have been developed for CNS diseases such as Alzheimer's disease (Yu et al., 2011).

1.1.4.5 Inhibitors of main drug efflux transporters of brain endothelial cells

Targeting the main efflux transporters of brain capillary endothelial cells, i.e. MRP, PgP and breast cancer resistance protein (ABCG2) is another mechanism that could increase the concentration of therapeutic agents in the CNS. Various different PgP inhibitors, e.g. HM30181A, cyclosporine A, valspodar, elacridir and zosuquidar have been shown to inhibit the efflux transporters leading to an increased uptake of chemotherapeutics into the CNS (Bauer et al., 2005, Joo et al., 2008a, Joo et al., 2008b). Probenecid - an inhibitor of MRP (Sun et al., 2001) and fumitremorgin C - an inhibitor of ABCG2 (Bakhsheshian et al., 2013) have demonstrated similar effects.

1.1.5 Preclinical models of brain metastases

To study different aspects of metastasis formation in the brain or to test targeted therapies in brain metastases, various different rodent models have been developed. Orthotopically transplanted primary tumours that give rise to metastatic foci in mouse models have been shown to produce animals that succumb to systemic disease before brain metastases can be reliably studied. This represents an important obstacle to the development of reliable CNS mouse models. Therefore, current models either use brain−seeking clones or directly implant tumours into the brain. The brain−seeking clones resulted from iterative experimental haematogenous dissemination to the brain in order to enrich for metastatic tumour burden in the CNS. Various experimental model systems for brain metastasis have been reported for multiple cancer types, including melanoma (Cranmer et al., 2005, Cruz-Munoz et al., 2008), lung carcinoma (Mathieu et al., 2004), and breast carcinoma (Price et al., 1990, Zhang et al., 1991, Price, 1996, Yoneda et al., 2001, Monsky et al., 2002, Chen et al., 2007). There are three different preclinical rodent animal models of brain metastasis including direct implantation, haematogenous metastasis and the spontaneous metastasis model.

Depending on the location of metastases in the CNS, two types of direct implantation model are distinguishable: leptomeningeal metastasis (Siegal et al., 1987, Schabet and Herrlinger, 1998, Izumi et al., 2002) and intraparenchymal implantation (Schackert et al., 1989, Broder et al., 2003, Carbonell et al., 2009). To establish leptomeningeal metastases, tumour cells are inoculated into the subarachnoid space, or indirectly to the cerebrospinal fluid space (CFS). This model is routinely used for longitudinal imaging studies with molecular or intravital microscopic imaging. In intraparenchymal models

tumour cells are directly injected into the brain parenchyma by stereotactic guidance. This model is used to study tumour growth inside the brain and can be extended via the placement of a cranial window. These experimental metastases models are relatively simple to execute, but they inevitably miss the initial steps of the metastatic cascade, and thus may not reflect the full clinical manifestation of the disease.

Haematogenous models of brain metastases can be achieved by intracardiac (Bos et al., 2009, Kienast et al., 2010) or intracarotid artery injection (Schackert and Fidler, 1988a, Schackert and Fidler, 1988b, Fidler et al., 1999) of tumour cells. For the intracardiac injection a single-cell tumour suspension is injected into the left ventricle of an animal, thereby allowing the cells to bypass pulmonary arrest and retention. This model exhibits a decreased reproducibility as tumour cells also disseminate to sites other than the CNS. By contrast, the intracarotid artery injection of metastatic cells directly into the internal carotid artery followed by permanent arterial ligation requires well-trained micro-surgical skills, but predominantly produces cerebral tumours and minimal non-cerebral metastases.

Spontaneous brain metastases models (Alterman and Stackpole, 1989, Yang et al., 1999, Cruz-Munoz et al., 2008) solely rely on the spontaneous formation of brain metastases following orthotopic implantation of cancer cells. This technique recapitulates all steps of the metastatic process, however, spontaneous brain metastases may require a longer duration to develop and can, therefore, require resection of primary tumours.

Based on the origin of implant, syngeneic and xenograft models can be distinguished. In syngeneic models the tumour tissue is derived from the same genetic background. Syngeneic animal models are reproducible and easy to handle and produce a transplant that is not rejected by the immune system of the host. However, these syngeneic tumours are not directly comparable with human tumours in terms of their tumour biology, and since the tumour cells are rodent, they express the mouse homologues of the desired targets.

Xenograft models mainly use existing human cancer cell lines that are implanted into immunodeficient mice (Teicher, 2006). As a result of *in vitro* manipulation, these cell lines have undergone genetic transformations (Daniel et al., 2009), which should be considered when used for research. Although different aspects of the process of cancer formation can be investigated with xenograft models, the stromal component of the tumour is rodent. Therefore, it lacks a functional immune system. Moreover, these models are more costly to run compared with syngeneic models (Teicher, 2006).

To understand all of the steps involved in the process of brain metastasis formation, the emergence of new animal models that more closely mimic the human disease is required. Using patient derived xenograft models instead of established cell lines could be one option. Further, preclinical research is also limited to only a few spontaneous models. Therefore, with the development of new models and imaging techniques, preclinical treatment strategies can become more focused on established brain metastases and provide more insight into brain metastasis formation.

1.2 Stem cell therapy

Stem cells can differentiate along different lineages and have the ability to self-renew (Potten and Loeffler, 1990). They can replenish dying cells and damaged tissues by multiplying via cell division and differentiating into a subset of cell types specific to its lineage. Therefore, they have an enormous regenerative and therapeutic potential. Cell therapy based on stem cells describes the process of introducing stem cells into a tissue to treat a disease with or without the addition of gene therapy. There are two major types of stem cells, embryonic stem cells and adult stem cells. So far, human embryonic stem cells (hESCs) were harvested from living embryos, therefore the use of hESCs involves stricter legal and ethical considerations (Green, 2007). However, the use of induced pluripotent stem cells (iPS) displays an alternative to the direct isolation of hESC (Takahashi and Yamanaka, 2006). The use of adult stem cells for therapy is less controversial and there have been advancements in clinical applications of neural, mesenchymal as well as haematopoietic stem cells recently.

1.2.1 Mesenchymal stem cells (MSCs)

Bone marrow-derived mesenchymal stem cells (MSCs), also called mesenchymal stroma cells or marrow stroma cells, can self-renew and display multi-lineage differentiation (Dennis et al., 2002). Friedenstein and his colleagues isolated and characterised these cells in a series of seminal studies in the 1960s and 1970s (reviewed in Friedenstein (1990)). MSCs originate from the mesoderm and exist in almost all tissues. Depending on specific *in vitro* conditions MSCs can differentiate along three different lineages, mesodermal, ectodermal and endodermal, into neuron, muscle, fat, bone, chondrocyte, islet and liver cells (Oishi et al., 2009). To identify MSCs, the expression of different markers can be used. These cells express α-smooth muscle actin, smooth muscle myosin heavy chain, nestin, Tuj-1, CD146, CD105, TGF-beta receptor, and various forms of integrins (reviewed in Ding et al. (2011)). The lack of surface markers, such as CD34, CD45, CD14, and class II major histocompatibility complex surface receptor (HLA-DR) is often used, to distinguish MSCs from HSCs (Pittenger et al., 1999). Further, Stro-1 is a surface marker specific for clonogenic MSCs (Simmons and Torok-Storb, 1991). A variety of tissues can be the source of MSCs, such as bone marrow, endometrium, adipose tissue, umbilical cord, or endometrial polyps (Ding et al., 2011). The ease of harvesting MSCs from these sources and the quantity that can be obtained makes them an attractive choice for experimental and clinical applications.

In 2013, more than 300 registered clinical trials worldwide evaluated the potential of MSC-based cell therapy (Wei et al., 2013). MSC therapy has been shown to be effective in the treatment of immune and non-immune diseases. The reparative effects, the wide tissue distribution and the multipotent differentiation of MSCs in many clinical and preclinical models suggests a critical role of MSCs in injury healing (reviewed in Wei et al. (2013)). Additionally, MSCs are capable of regulating immune responses. This ability has been utilised by patients receiving bone marrow transplantation (BMT) and suffering from graft-versus-host disease (GvHD), where the effect was successfully reversed with MSC administration (Muller et al., 2008, Prasad et al., 2011). Further, since tumours continuously generate various inflammatory cytokines and are therefore regarded as wounds (Dvorak, 1986), therapies including MSC administration have been explored. MSCs have been shown to migrate to tumours and adjacent tissue sites following *de novo* mobilisation or exogenous administration (Spaeth et al., 2008). The role of MSCs as cellular delivery vehicles to treat different types of cancer, such as melanoma and Kaposi's sarcoma has been explored by many research groups in preclinical studies (Studeny et al., 2002, Elzaouk et al., 2006, Khakoo et al., 2006). Moreover, the potential of mesenchymal stem cells as delivery vehicles to treat different CNS tumours has also been explored recently. MSCs have been used as cellular delivery vehicles of therapeutic genes to treat glioma-bearing rodents (Bexell et al., 2012, Nakamura et al., 2004, Nakamizo et al., 2005) and as vehicles for the delivery of nanoparticles to glioma to enhance their tumouricidal effects (Roger et al., 2010). Following intratumoural injection, MSCs migrated efficiently within the tumour and did not proliferate. However, systemically administered MSCs exhibited only insufficient homing to the brain (Bexell et al., 2012).

1.2.2 Neural stem cells (NSCs)

NSCs have the potential to self-renew, the competence for *in vivo* regeneration and possess neural tripotency, i.e. the capability to give rise to all of the major neural lineages: neurons, astrocytes and oligodendrocytes (Conti and Cattaneo, 2010). These cells are immature cells present in the developing and adult CNS. NSCs also account for the limited regenerative potential in the adult brain. In the adult CNS, NSCs reside in defined regions called neurogenic niches (Casarosa et al., 2014). There they sustain their multipotency and regulate the balance between self-renewal and differentiation by symmetrical or fate-committed asymmetric divisions, respectively (Fuentealba et al., 2012). The phenotype of NSCs has not been completely determined. Preliminary

characterisation studies defined potential NSC subsets via the expression CD133 and lack of expression of surface markers such as CD34 and CD45. However, the distinct subset of human foetal CNS cells with the phenotype $CD133^{+}/5E12^{+}/CD34^{-}/CD45^{-}/CD24^{-/lo}$ has been shown to form neurospheres in culture, to initiate secondary neurosphere formation, and to differentiate into neurons and astrocytes (Reynolds and Rietze, 2005). Further markers, such as Nestin, Sox 1/2 and Musashi 1, have also been used to identify NSCs (summarised in Jandial et al. (2008)). Sources of neural cells with the properties of stem cells can be embryonic, neonatal, and adult rodent and human CNS using several different *in vitro* expansion methods (Conti and Cattaneo, 2010). Although there have been a variety of protocols described for NSC purification, generation and expansion over the last two decades, the identification of the best sources for NSCs and the optimisation of protocols to stably expand them clonally *in vitro* remains a major goal of NSC research (Casarosa et al., 2014).

The capacity of NSCs to divide and differentiate into cells of the CNS under appropriate *in vitro* conditions demonstrates the potential for clinical application for various CNS diseases. On the one hand, NSC therapy could be used to treat neurodegenerative disorders, such as Huntington's disease (HD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD) or non-degenerative conditions, such as spinal cord injury and stroke. Zhu et al. (2009) demonstrated an improved behaviour in PD rat models following direct injection of NSC to the diseased brain area. NSCs were also used as delivery vehicles for glial cell line-derived neurotrophic factor (GDNF) in HD mouse models, which led to the prevention of degeneration and an improved behaviour of the mice (Pineda et al., 2007). Further preclinical experiments using NSC therapy could demonstrate therapeutic efficacy for different diseases such as AD, ALS, stroke and spinal cord injuries (Zhang et al., 2014, Zhang et al., 2015, Hwang et al., 2009, Sun et al., 2010, Ormond et al., 2014).

Furthermore, several research groups have detected NSCs near tumour foci far from the original brain transplant site following transplantation into animal models of brain neoplasia (Aboody et al., 2000, Benedetti et al., 2000). Aboody et al. used NSCs as delivery vehicles of the enzyme cytosine deaminase, which converts a non-toxic prodrug into a chemotherapeutic agent. This suicide gene therapy demonstrated therapeutic efficacy in murine glioma models. The corresponding clinical trial recruited participants with recurrent brain tumours and has since been completed, but the study results are not yet published (ClinicalTrial.gov, 2015). Recently, it has also been shown that the tumour-homing ability of NSCs is not limited to glioma lesions: NSCs can also target melanoma (Aboody et al., 2006), breast cancer brain metastases (Joo et al., 2009), as well as

disseminated neuroblastomas (Sims et al., 2009) and intracerebral medulloblastomas (Kim et al., 2006). However, intratumoural injection has additional risks for patients and primary autologous NSCs cannot be isolated in the quantities required for the therapy.

1.2.3 Haematopoietic stem cells (HSCs)

The mammalian blood system with more than ten distinct mature cell types derives from one specific cell type, haematopoietic stem cells. HSCs possess the ability of self-renewal and multi-potency. These cells can differentiate into all types of blood cells. They can give rise to myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells) and lymphoid lineage cells (T-cells, B-cells, NK-cells). Thus, they can be used to treat blood and immune disorders. HSCs balance self-renewal and differentiation, to maintain the HSC pool throughout life while continuously providing differentiated progenitors, as mature blood cells are typically short-lived. HSCs reside primarily in the bone marrow but also circulate in the periphery. The cell surface expression of the CD34 antigen was the first marker used to identify human HSCs, because its expression is down-regulated as cells differentiate into more abundant mature cells (Andrews et al., 1989). The first markers found to be specifically upregulated in murine HSCs were Sca-1 (Spangrude et al., 1988) and c-kit (Ogawa et al., 1991). However, since then more markers have been found to be specifically up- or down-regulated in human and murine HSCs (summarised in Figure 1-4). In 1963, Siminovitch et al. provided the first evidence for the existence of HSC in bone marrow and thereby defined the hallmark properties of HSCs (Siminovitch et al., 1963). Following administration of HSCs into lethally irradiated mice, HSCs were able to reconstitute the haematopoietic system. They further demonstrated the self-renewal capacity of HSCs by performing serial transplantations. This experiment marked the beginning of modern-day stem cell research. The following chapters of this thesis will emphasise further characteristics of these cells and advances of HSC therapies.

1.2.3.1 Haematopoiesis of the mammalian blood system

There are more than ten distinct mature haematopoietic cell types including myeloid cells (monocyte/macrophage and granulocytes), mast cells, T- and B- lymphocytes, natural killer (NK) cells, dendritic cells (DC), red blood cells (erythrocyte) and megakaryocytes/platelets in the mammalian blood system. These diverse mature cell

types are derived from a common progenitor cell, i.e. HSC. Starting with the multipotency of HSC, there is a hierarchical structure in haematopoietic development in which this multipotency is progressively restricted. Initially, HSCs give rise to multipotent progenitors (MPPs) that still possess full-lineage differentiation potential, but have lost the ability to self-renew (Morrison and Weissman, 1994, Christensen and Weissman, 2001). This population of murine MPPs has been found to be heterogeneous (Adolfsson et al., 2005, Forsberg et al., 2006, Arinobu et al., 2007). MPPs progress to oligopotent progenitors, the common myeloid progenitor (CMP) (Akashi et al., 2000) or the common lymphoid progenitor (CLP) (Kondo et al., 1997, Karsunky et al., 2008, Serwold et al., 2009). These oligopotent progenitors then give rise to all the lineage-committed effector cells of the hematopoietic system. CMPs can give rise to megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/ macrophage progenitors (GMPs) (Nakorn et al., 2003, Pronk et al., 2007), which then differentiate to platelets, erythrocytes, granulocytes and macrophages. CLPs can differentiate to T- and B-lymphocytes, NK cells and dendritic cells (DCs). It has been shown that the three different subsets of DCs ($CD8\alpha^+$DC, $CD8\alpha^-$DC, and plasmacytoid DC) can be derived either from CMP and CLP (Traver et al., 2000, Manz et al., 2001a, Manz et al., 2001b).

This multi-tiered hierarchy resembles mouse and human haematopoiesis (Figure 1-4). The hierarchical structure and the balance of the enormous production of mature white blood cells (Ogawa, 1993) and the precise maintenance of the stem cell homeostasis (Rossi et al., 2007) is characteristic of haematopoiesis.

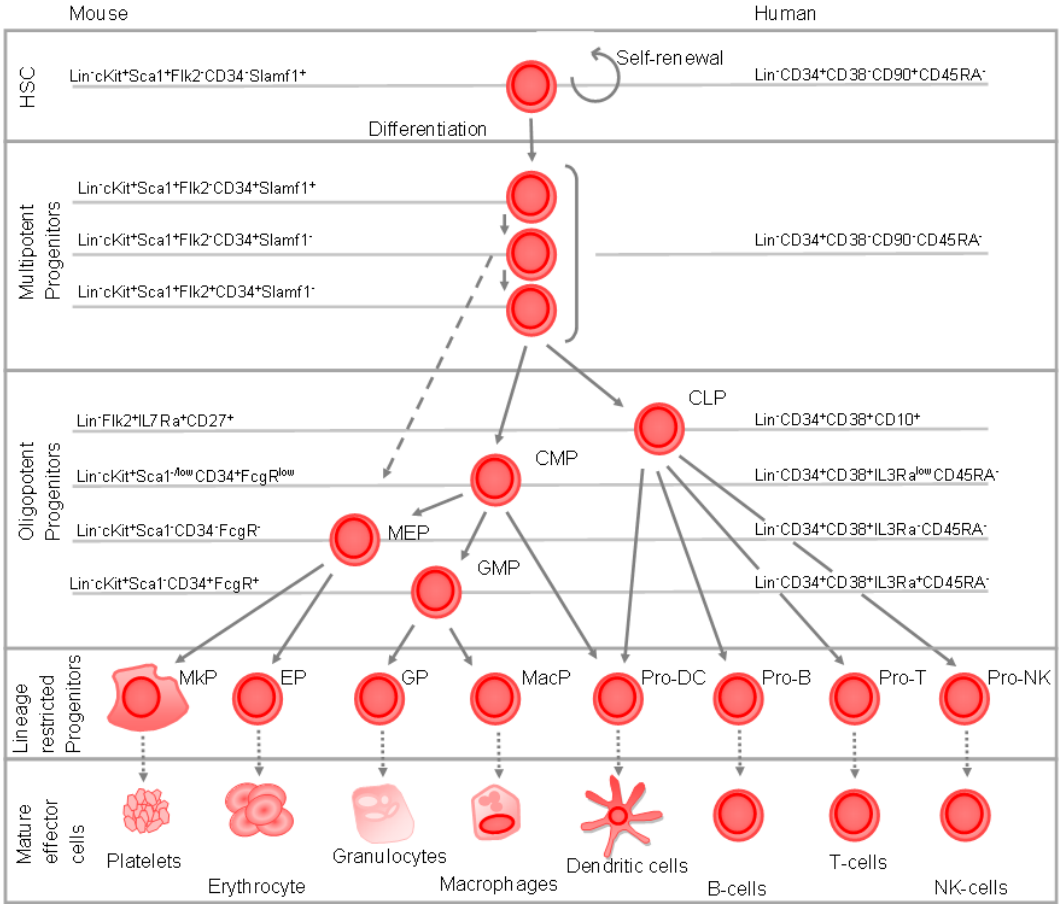


Figure 1-4: Model of the haematopoietic hierarchy (adapted from Seita and Weissman (2010)). At the top of the hierarchy the HSC has self-renewal and multipotency capacity. A HSC first loses self-renewal ability, then multi-lineage potential, as it becomes a mature functional cell of a certain lineage throughout the differentiation process. The cell surface phenotype of each population is shown for the mouse and human systems. CLP: common lymphoid progenitor, CMP: common myeloid progenitor, DC: dendritic cell, EP: erythrocyte progenitor, GMP: granulocyte/macrophage progenitor, GP: granulocyte progenitor, HSC: hematopoietic stem cell, Lin: lineage markers; antigens specific to terminally differentiated blood cells, MacP: macrophage progenitor, MEP: Megakaryocyte/erythrocyte progenitor, MkP: Megakaryocyte progenitor, NK: natural killer.

1.2.3.2 Sources of HSCs

HSCs can be harvested from different sources, including bone marrow, peripheral blood and umbilical cord blood.

The bone marrow consists of various different cell types, including HSCs, stromal cells, stromal stem cells, blood progenitor cells as well as mature, or maturing, lymphocytes. Typically bone marrow transplants are performed by anaesthetising the stem cell donor, puncturing a bone, which is normally a hipbone and drawing out the bone marrow cells with a syringe (Shah et al., 2015).

However, HSC isolation from the bone marrow is now rare in the clinical setting and harvesting HSCs from the peripheral, circulating blood is the preferred method (Lapierre et al., 2000). HSCs are mobilised from their marrow niche by injecting the cytokine granulocyte-colony stimulating factor (G-CSF) a few days prior to the cell harvest. $CD34^+$ cells will be specifically collected from blood, whereas all $CD34^-$ cells will be re-injected to the donor. This method of collecting stem cells produces minimal pain, needs no anaesthesia and no hospital stay. It is also known that patients receiving peripherally harvested cells have higher survival rates than bone marrow recipients, as twice as many HSCs can be collected and the engraftment takes more rapidly (Negrin et al., 2000, Childs et al., 2000).

Umbilical cord blood is another source of HSCs. The stem cell concentration is at least 100-fold greater than in adult peripheral blood. This tissue can be harvested at birth and cryopreserved at cord blood banks. Umbilical cord blood recipients are usually children (Laughlin, 2001).

Further sources of human HSCs are the foetal haematopoietic system (Labastie et al., 1998, Gallacher et al., 2000) and embryonic stem cells (Itskovitz-Eldor et al., 2000, Shamblott et al., 2001, Ng et al., 2008). However, these sources have only been used for research and not for clinical applications.

Murine HSCs can be isolated from bone marrow, spleen and foetal liver and are used for research purposes. Mature cells expressing lineage antigens (Lin) specific to terminally differentiated blood cells of these tissues can be removed by methods such as magnetic activated cell sorting (MACS).

1.2.3.3 HSC transplantation (HSCT)

HSC transplantation is a routinely performed clinical procedure, that offers a potential cure for haematologic cancers such as leukaemia, lymphoma as well as myeloma and other haematologic disorders including primary immunodeficiency, aplastic anaemia and myelodysplasia (Copelan, 2006). A successful engraftment reconstitutes the entire haematopoietic system of an individual and thereby replaces the diseased haematopoietic cells of the patient. There are two different graft types of HSCT, i.e. allogeneic and autologous. For an autologous HSCT the patient's own stem cells are used, whereas stem cells from a donor are used for allogeneic transplantation. To be a suitable donor for an allogeneic HSCT, the human leukocyte antigens (HLAs) on the surface of the haematopoietic cells have to match (Gluckman et al., 2004, Copelan,

2006). There are two categories of HLA genes, Type I and Type II. If there is mismatch of Type-I genes, i.e. HLA-A, HLA-B, or HLA-C the risk of graft rejection is increased. A mismatch of an HLA Type II gene, i.e. HLA-DR, or HLA-DQB1 can cause the risk of GvHD. Even small genetic disparities, such as one single DNA base pair mismatch, can also lead to side effects. Therefore, the exact DNA sequence of all five HLA genes has to be identified to find a suitable donor.

Before the HSCT, the recipient receives a conditioning therapy to eradicate stem cells in the bone marrow and suppress the recipient's immune response (Copelan, 2006). The conditioning regimen can either be myeloablative or non-myeloablative depending on the type of disease. The myeloablative regimen is often a combination of chemotherapeutic and radiation therapy. The non-myeloablative transplantation also uses a combination of chemotherapeutic and radiation therapy, but in doses too low to completely ablate all bone marrow cells of the recipient. This regimen lowers the risks of serious infections and transplant-related mortality (Alyea et al., 2006). However, the non-myeloablative regimen entails an increased risk of cancer relapse.

After the HSCs have been administered, patients are given colony-stimulating factors to shorten duration of post-transplantation leukopenia and prophylactic anti-infective drugs (Shah et al., 2015). Allogeneic HSCT recipients will additionally be administered prophylactic immunosuppressants to prevent a donor T cell-induced GvHD. Engraftment typically occurs 10 to 20 days post HSCT with bone marrow stem cells, but earlier with peripheral blood stem cells, and is defined by an absolute neutrophil count.

1.2.3.4 HSC gene therapy

Haematopoietic stem cell gene therapy combines the capability of haematopoietic stem cells to replace the entire blood and immune system of an individual with the capacity for long-term replacement of gene copies using gene therapy vectors for integration. Therefore, it is an attractive treatment option for many diseases, including genetic disorders, haematologic conditions and immunodeficiencies. Thereby, HSC therapy greatly benefits from the clinical experience of standard blood and bone marrow transplantation. Whereas safe allogeneic HSCTs require a HLA-matched donor, individuals receiving HSC therapy could potentially donate their own cells. Therefore, there is no risk of GvHD and it has been shown that immune reconstitution occurs more rapidly following an autologous transplant with genetically engineered cells (Mukherjee and Thrasher, 2013). Current HSC gene therapies are based on *ex-vivo* transfer of a

therapeutic transgene via viral vectors to patient-derived autologous HSCs followed by transplantation back to the patient.

Preclinical therapies

One option to employ HSC gene therapy in cancer is to target the immunosuppressive tumour microenvironment. Escobar et al. (2014) developed a lentiviral vector for interferon-α (IFN-α) under the regulation of the Tie2 promoter, which is specifically active in TEMs. Type I IFNs are cytokines involved in innate and adaptive immunity that have been shown to promote anti-tumour immune responses (Dunn et al., 2005). Inhibition of tumour progression was achieved in mouse breast cancer models following transplantation of HSCs transduced with the Tie2/IFN-α lentiviral construct. The transgene expression in tumour-infiltrating monocytes/macrophages reprogrammed the tumour microenvironment toward more effective dendritic cell activation and immune effector cell cytotoxicity.

Another approach to target the immunosuppressive tumour microenvironment has been developed by Shah et al. (2002). The retroviral-mediated HSC gene therapy that uses the transgene of the dominant-negative mutant of TGF-β receptor II (TβRIIDN) has been shown to efficiently suppress tumour metastasis. TGF-β is an immunosuppressive cytokine and the dominant-negative receptor is capable of binding all three TGF-β isoforms without mediating signal transduction. However, systemic expression of this receptor leads to multi-organ inflammation and the production of autoantibodies (Gorelik and Flavell, 2000). To avoid cellular dysfunctions, the gene therapy approach was modified. The heat-shock protein 70 (Hsp70) promoter, highly active within the tumour microenvironment, was used to regulate the expression of TβRIIDN. A massive anti-tumour response was observed in glioma-bearing mice treated with the Hsp70/TβRIIDN HSC gene therapy (Noyan et al., 2012).

Relaxin (Rlx) is a peptide hormone that can mediate the degradation of tumour stroma proteins. Li et al. (2009) developed a gene therapy for breast cancer using genetically modified HSCs. It was shown that the transplantation of mouse HSCs transduced with an Rlx-expressing lentivirus vector delayed tumour growth in a mouse model of breast cancer. The Rlx-mediated degradation of tumour stroma contributed to an increased access of infiltrating immune cells to their target tumour cells. A significant delay of tumour growth could be observed when this approach was combined with trastuzumab therapy (Beyer et al., 2011).

HSC gene therapy can also be used to correct mutations of genes in haematopoietic cells. Mutations in the Artemis gene cause a complete absence of T- and B- lymphocytes in patients, which can lead to a radiosensitive severe combined immunodeficiency (RS-SCID). As matching HSCT donors might not always be available for patients, alternative therapeutic approaches such as gene therapy have been investigated. The Artemis-transduced HSCs were transplanted into irradiated Artemis knockout mouse Art(-/-) (Benjelloun et al., 2008). This restored a stable and functional T- and B-cell repertoire that was comparable to that of control mice. Thereby, this therapy provides a basis for supporting the gene therapy approach in Artemis-deficient SCID.

Studies have also looked at whether bone marrow toxicity from chemotherapeutic agents can be prevented via cytostatic drug resistance gene therapy. To this end, HSCs that were transduced with the multidrug resistance (MDR) 1 gene were administered into tumour-bearing mice (Guo and Jin, 2006), then, by increasing the proportion of bone marrow cells expressing the chemo-protective gene, the influence on bone marrow toxicity from chemotherapy was determined. The white blood cell (WBC) counts revealed that the mice that had received gene-transduced cells showed a significant increase in WBCs count compared with naïve counterparts. This cytostatic drug resistance gene therapy can provide some degree of chemo-protection, which could allow an increase in chemotherapy dose high enough to kill tumour cells.

Another gene therapy-mediated approach was developed to inhibit tumour-induced neovascularisation. Murine bone marrow cells were transduced with a retroviral vector encoding the angiogenesis inhibitor foetal liver kinase-1 (Flk-1), which is the soluble truncated form of the vascular endothelial growth factor receptor-2 (Davidoff et al., 2001). One of the primary tumour-expressed endothelial mitogens is VEGF3 (Dvorak et al., 1995), and the activity of this ligand can be inhibited by a soluble truncated form of one of its receptors, Flk-1 (Millauer et al., 1996, Lin et al., 1998). After transplantation with tsFlk-1-expressing bone marrow cells the tumour growth in mice was significantly inhibited compared with tumour growth in control-transplanted mice. Further IF analysis of tumour tissue revealed transgene expression in endothelial cells of the tumour-induced neovasculature. These data show that these cells were derived, at least in part, from bone marrow precursors. This HSC gene therapy has demonstrated therapeutic efficacy by targeting tumour neovasculature, as bone marrow-derived endothelial cell precursors seem to be recruited in the process of tumour-induced angiogenesis.

Cinical Trials

Several HSC therapies have already been clinically tested for various immune deficiency disorders. Initial clinical trials, using genetically engineered HSCs by retroviral transduction for adenosine deaminase (ADA) deficiency, have shown little clinical benefit (Blaese et al., 1995). The enzyme ADA catalyses the breakdown of toxic metabolites of purine degradation, which affects lymphocyte development and function in pathological state. Patients with ADA are currently treated with HSCT and enzyme replacement therapy (ERT). Further trials optimised the transduction efficiency of injected HSCs, which allowed for termination of ERT in 75% of the treated patients following the reconstitution of genetically corrected autologous cells (Aiuti et al., 2009, Candotti et al., 2012, Gaspar et al., 2011b).

Mutations in the common gamma chain, present in several cytokine receptors, can lead to a radical interference with immune cell development resulting in X-linked severe combined immunodeficiency (X-SCID). To replace the mutated gene, genetically engineered HSCs were given to patients and this led to successful reconstitution in 17/19 patients. However, in five of these patients T cell acute lymphocytic leukaemia (T-ALL) has been diagnosed, following insertional transactivation of proto-oncogenes after retroviral transduction (Hacein-Bey-Abina et al., 2010, Gaspar et al., 2011a).

There have also been HSC therapy clinical trials for patients suffering from chronic granulomatous disease (CGD). This disease can be caused by the mutation of one of five genes that are responsible for the generation of reactive oxygen species (ROS) in phagocytic cells. Several studies showed improved patient outcome after genetically engineered HSCs were administered to correct the mutation (Ott et al., 2006, Bianchi et al., 2009, Kang et al., 2010). However, retroviral integration site-distribution analysis showed activating insertions near proto-oncogenes MDS1-EVI1, PRDM16 or SETBP1 and clonal outgrowth of gene marked cells was also observed in some patients (Ott et al., 2006).

Notably, a clinical study by Cartier et al. has previously demonstrated the use of HSCs to deliver therapy to the brain (Cartier et al., 2009). This phase I/II clinical trial for the treatment of X-linked adrenoleukodystrophy (ALD) uses an autologous $CD34^+$ HSC transplantation. A mutation of the X-linked peroxisomal transporter protein ABCD1 causes the disruption of transport and subsequent breakdown of very long-chain fatty acids (VLCFAs), leading to abnormally high levels of these fats in the body and ultimately causing demyelination. The mutation responsible for ALD is corrected via gene therapy

using a lentiviral vector to transduce patients' HSCs. This produces corrected microglial cells and a successful therapy for this CNS disease.

Further HSC gene therapies to correct genetic mutations of immunodeficiencies have been shown to be successful. Following HSC gene therapy of 10 conditioned Wiskott-Aldridge syndrome (WAS) patients there has been sustained engraftment of cells sufficient to correct disease manifestations. However, four patients developed leukemia following transactivation of proto-oncogenes (Boztug et al., 2010). A clinical trial for metachromatic leukodystrophy (MLD) patients showed an improved outcome following engraftment of genetically engineered HSCs with no evidence of vector mediated toxicity (Biffi et al., 2013).

Though therapeutic efficacy could be demonstrated for all HSC gene therapy trials, several trials have reported side effects caused by insertional mutagenesis following LTR-γ-retrovirus transduction. The use of self inactivating (SIN)-retroviral and -lentiviral vectors can significantly reduce the risk of insertional mutagenesis. SIN vectors feature a deleted promoter region in the 3′ long terminal repeats (LTR), which results in integrated LTRs lacking the promoter activity. Several clinical studies for HSC gene therapy using SIN vectors are currently ongoing (summarised in Mukherjee and Thrasher (2013)). To date no transformation events have been reported with SIN vectors (Bigger and Wynn, 2014).

1.3 Gene therapy against cancer

Gene therapy also displays an alternative to current treatment options for cancer, which is demonstrated by various current clinical trials (Sangro et al., 2010, Kaufman and Bines, 2010, Nokisalmi et al., 2010, Sterman et al., 2010, Anwer et al., 2010, Guan et al., 2011). Moreover, gene therapy has the potential to target cancer cells while sparing normal tissues. This technique can be useful for recurrent cancer disease as well as in the adjuvant setting.

The goal of gene therapy is to introduce a functional gene into target cells. The inserted genetic material can either encode a therapeutic molecule to target diseased cells or encode a functional gene to replace a genetic defect that caused a dysfunction. Different strategies can be used in cancer gene therapy including mutation correction, enhancement of the immune response against tumour cells, RNA interference, anti-angiogenic and enzyme prodrug therapies (Vassaux and Martin-Duque, 2004).

Therapeutic transgenes have been used to correct the primary genetic defect in many cancer cells, i.e. p53. Restoring the p53 function in cancer cells via gene therapy successfully induced apoptosis in cancer cells and thus tumour regression in patients (Swisher et al., 2003). A gene therapy using TNF-α, a cytokine with potent anti-cancer properties and high systemic toxicity, demonstrated therapeutic efficacy in patients with pancreatic, rectal cancer and melanoma (Senzer et al., 2004, McLoughlin et al., 2005, Herman et al., 2013). Rexin-G is an agent that contains the cytocidal cyclin G1 construct that accumulates preferentially in tumour cells to block the action of cyclin G1 and initiate cell death. Metastatic pancreatic cancer patients experienced tumour growth arrest following Rexin-G gene therapy treatment (Chawla et al., 2010). Another strategy to use gene therapy in cancer is to enhance the immune response via the expression of the cytokine IL-12 (Tahara and Lotze, 1995). However, in experimental models tumour regression was only observed when used in combination with other drugs (Rakhmilevich et al., 2004, Denies et al., 2014).

Other cancer treatments involving gene therapy rely on the potential for prodrug activation. These enzyme prodrug therapies are the most commonly used gene therapy options for cancer, and are explained in more detail in the following section.

1.3.1 Enzyme prodrug gene therapy systems

In an enzyme prodrug therapy a viral or bacterial transgene is introduced into a tumour cell and this transgene allows the conversion of a non-toxic compound into a lethal drug. Enzyme prodrug therapy has been successfully used in a large number of *in vitro* and *in vivo* studies. Its application in cancer patients has not reached clinical significance, although recent reports on preclinical cancer models demonstrated the huge potential of this strategy when used in combination with new therapeutic approaches (Freytag et al., 2007, Liu et al., 2007).

It is a two-step approach, whereby a drug-activating enzyme expressed by cancer cells or cells of the tumour microenvironment convert the systemically administered non-toxic prodrug into the active drug within the tumour. The enzyme and the prodrug must meet certain requirements to make it a successful therapy. The prodrug converting enzyme should either be of non-human origin or a human enzyme only expressed at low concentrations in normal tissue (Rainov et al., 1998, Xu and McLeod, 2001). In addition, the enzyme should be expressed abundantly within the tumour and have a high catalytic activity (Niculescu-Duvaz et al., 1998). Furthermore, the prodrug should be a good

substrate for the expressed enzyme in the tumour tissue, not be activated by endogenous enzymes in non-tumour tissues and have minimal toxicity prior to activation. The activated toxic drug is expected to be highly diffusible or actively taken up by adjacent cells in order to kill cancer cells via a bystander killing effect (Niculescu-Duvaz et al., 1998). Furthermore, to induce the bystander effect, the half-life of the activated drug should be of a sufficient length but short enough to prevent the drug from leaking into the systemic circulation (Niculescu-Duvaz et al., 1998). A large number of enzyme prodrug systems have been developed and some of these are discussed in detail below.

1.3.1.1 Cytosine deaminase/5-Fluorouracil (CD/5-FC)

The cytosine deaminase system was first described as a negative selection system for experimental studies and in treatments using gene transfer techniques (Elion et al., 1977). Cytosine deaminase is expressed by bacteria and yeast but is absent in mammalian cells. The enzyme normally catalyses the deamination of cytosine to uracil and ammonia. However, this enzyme can also convert the prodrug 5-fluorocytosine (5-FC) to 5-Fluorouracil (5-FU), an important drug used in conventional chemotherapy. 5-FU can act via 3 different pathways to induce cell death, including thymidylate synthase inhibition, formation of (5-FU) RNA and of (5-FU) DNA complexes (Niculescu-Duvaz and Springer, 1997). This is because it can be transformed by cellular enzymes into different potent pyrimidine anti-metabolites including 5-Fluoro-2'-deoxyuridine-5'-monophosphate (5-FdUMP), 5-Fluorouridine-diphosphate (5-FUDP) and 5-Fluorouridine-triphosphate (5-FUTP). 5-FdUMP is an irreversible inhibitor of thymidylate synthase, resulting in thymidine starvation and inhibition of DNA synthesis. 5-FUDP is further processed to 5-FdUTP which can be incorporated into DNA and lead to DNA damage and apoptosis. 5-FUTP can also be incorporated into RNA, substituting for UTP and inhibiting RNA processing (Biasco et al., 2012). Therefore, this enzyme prodrug therapy minimises the normal systemic side effects of 5-FU therapy, and maximizes the local anti-tumour effect.

The CD/5-FC system can be further improved by the inclusion of the uracil phosphoribosyltransferase (UPRT) gene which allows the conversion of 5-FU to 5-fluorouridine monophosphate, the first step of its pathway to activation (Kanai et al., 1998). Interestingly, this CD-UPRT/5-FC system has been shown to be effective in 5-FU-resistant human primary cancer cells (Richard et al., 2007).

Promising *in vivo* anti-tumour activity of the CD/5-FC therapy has been demonstrated in different preclinical models, including fibrosarcomas (Mullen et al., 1994), carcinomas

(Huber et al., 1993, Kanai et al., 1997, Bentires-Alj et al., 2000), gliomas (Ichikawa et al., 2000) and metastatic tumours of different origins (Consalvo et al., 1995, Topf et al., 1998). A number of clinical trials have been reported using the CD/5-FC system, although its application in the clinic has been limited (Pandha et al., 1999, Nemunaitis et al., 2003, Freytag et al., 2007).

1.3.1.2 Herpes simplex virus thymidine kinase/ganciclovir (HSV TK/GCV)

The most intensively studied enzyme prodrug system is herpes simplex virus thymidine kinase (HSV TK) with the prodrug ganciclovir. The enzyme HSV TK catalyses the phosphorylation of ganciclovir (GCV) to ganciclovir monophosphate (Fillat et al., 2003), which is converted by cellular kinases to di- and triphosphate derivatives. Ganciclovir triphosphate is an analogue of deoxyguanosine triphosphate and can be incorporated into DNA by cellular DNA polymerase and cause DNA chain termination and apoptosis (Balfour, 1999, Wang et al., 2004, Ketola et al., 2004).

The therapeutic efficacy of the HSV TK/GCV gene therapy system has been demonstrated in several experimental carcinoma models, including leukaemia (Bondanza et al., 2011), glioma (Staquicini et al., 2011), and bladder cancer (Tang et al., 2009). This has led to the application of the HSV TK/GCV system in a number of clinical trials involving different types of cancer, including glioma (Voges et al., 2003), prostate cancer (Nasu et al., 2007), hepatocellular carcinoma (Li et al., 2007), as well as head and neck cancer (Xu et al., 2009).

The limitations of this system include the potential immunogenicity of the viral enzyme, and the requirement for active mitosis to induce cell death (Dong and Woraratanadharm, 2005). Therefore, other enzyme prodrug gene therapy systems would represent better options with fewer limitations.

1.3.1.3 Carboxyl esterase/irinotecan (CE/CPT-11)

Another established enzyme prodrug gene therapy system combines the enzyme carboxyl esterase with the prodrug irinotecan. Irinotecan (CPT-11) is a chemotherapy prodrug which is cleaved by CE to the potent anti-tumour agent 7-ethyl-10-hydroxycamptothecin (SN-38). SN-38 is an inhibitor of topoisomerase I activity (Kawato et al., 1991), which causes accumulation of double-strand DNA breaks in actively

dividing cancer cells that results in the inhibition of both DNA replication and transcription. Anti-tumour efficacy of this system in a preclinical lung cancer model was demonstrated (Kojima et al., 1998). However, further preclinical and clinical applications of this system for other types of cancer were not performed. This might also be due to the fact that the enzyme is found in a variety of tissues, including serum, liver and the intestine (Kaneda et al., 1990), which could lead to non-specific enzyme prodrug activity.

1.4 Viral DNA delivery

Because viruses have evolved natural mechanisms to deliver their genomes into cells, they are excellent vectors to deliver foreign DNA. Usually, a virus penetrates into the nucleus of the host cell, exploits the cellular machinery to express its own genetic material and replicate it, and then spreads to the other cells (Kay et al., 2001). A variety of viruses have been used to deliver therapeutic genes into cell nuclei. In order to use a virus as a vector to transfer a gene, it is modified by genetic engineering. The therapeutic gene replaces the pathogenic part of the virus (Bouard et al., 2009), while the virus retains its non-pathogenic structures that allow cell infection (Kay et al., 2001). The resulting non-pathogenic virus carrying the therapeutic gene is called a viral vector. The first vectors based on retroviruses were developed by Mann et al. (1983) and have been successfully used in gene therapy trials more than 20 years ago (Anderson et al., 1990, Blaese et al., 1993). Due to their high transduction efficiency *in vivo*, viral vectors are the most attractive choice to transfer genes (Munier et al., 2005). However, viral vectors also have major drawbacks, such as acute immune responses, the expensive and difficult production of the viral vectors in quantities needed and the size of the gene delivered by the virus is also limited (Templeton, 2002, Munier et al., 2005). The most commonly used viral vectors for gene therapy derive from adenoviruses, retroviruses, vaccinia virus, adeno-associated viruses, herpes simplex virus and lentiviruses (Ibraheem et al., 2014). The choice of viral vector depends on several parameters such as the characteristics of the cancer type and the therapeutic strategy.

1.4.1 Lentivirus

Recently, research has focussed on lentiviruses as transgene delivery vehicles. Lentiviruses, such as the human immunodeficiency virus (HIV), are complex retroviruses and belong to the family of retroviridae, which are single-stranded RNA spherical viruses.

The lentiviral particle is lipid-enveloped and measures around 80 to 100 nm (Vogt and Simon, 1999).

The lentiviral genome is organised in the gag, pol and env gene. The structural proteins are encoded by gag, whereas the pol gene encodes the enzymes that accompany the ssRNA, i.e. reverse transcriptase, integrase and protease. The reverse transcription of viral RNA to DNA is catalysed by the reverse transcriptase and the integrase mediates the integration of the proviral DNA into the host genome. The protease enzyme is involved in gag-pol cleavage and virion maturation (Katz and Skalka, 1994). The viral envelope glycoproteins are encoded by the env gene. Additionally, there are accessory genes that regulate the viral gene expression, assembly and replication (Coil and Miller, 2004). Furthermore, there are cis-acting sequences, such as two LTRs, in the retroviral genome that are required for gene expression, reverse transcription and integration into the host chromosomes. Further, the polypurine tract (PPT) is enclosed in the viral genome, which is the site of the initiation of the positive strand DNA synthesis during reverse transcription (Charneau et al., 1992). Another important component is the packaging signal (psi or ψ), which is required for the specific RNA packaging into newly formed virions (Watanabe and Temin, 1982).

1.4.2 Lentiviral vectors

Third generation lentiviral particles are generated by the co-transfection of 4 plasmids in human embryonic kidney (HEK) 293T cells (Naldini et al., 1996). These plasmids are called packaging plasmids, the transfer plasmid and the envelope-encoding plasmid. Together, these plasmids only represent a fraction of the lentiviral genome, lacking all genes that are not critical for efficient gene transfer (Dull et al., 1998). Moreover, this vector system is designed to produce virus particles that are replication deficient, which means that after transgene delivery the particles are unable to infect their host (Escors and Breckpot, 2010). Additionally, these viruses are able to transduce non-dividing cells (Bukrinsky et al., 1993). Many gene therapy strategies target highly differentiated cells. Therefore, the characteristics of lentiviruses give them an advantage over other viruses. Currently, there are 114 clinical trials worldwide using lentiviral vectors for gene delivery (Wiley.com, 2015).

The development of lentiviral vector systems is characterised by modifications of the packaging plasmids to improve safety (summarised in Escors and Breckpot (2010)). The components of the third generation lentiviral vectors are shown in Figure 1-5.

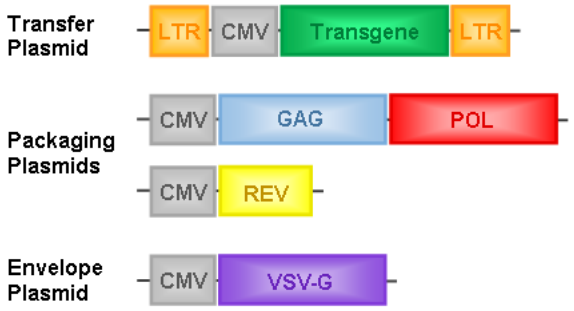


Figure 1-5: The third generation lentiviral vectors. The transgene sequence is located on the transfer plasmid and flanked by the cis-acting LTRs. The packaging plasmids contain sequences of structural proteins and enzymes. The envelope plasmid encodes the vesicular stomatitis virus-glycoprotein, which is incorporated in the viral lipid envelope. Expression of these genes can be regulated by the cytomegalovirus (CMV) promoter.

1.4.3 Lentiviral packaging and transduction

Following transfection of the lentiviral plasmids, the packaging cells produce proteins according to the genetic information of the four lentiviral vectors to assemble the virus particle which is then released into the cell supernatant (Figure 1-6). The packaging plasmids comprise gag, pol, and rev. The latter encodes a post-transcriptional regulator necessary for efficient gag and pol expression. The envelope plasmid encodes a glycoprotein, which is incorporated in the viral lipid envelope and allows pseudotyping of the lentiviral particles. The most used glycoprotein is the vesicular stomatitis virus-glycoprotein (VSV-G), which confers a very broad tropism for human and non-human cells (Yee et al., 1994). The transfer plasmid contains the transgene which is flanked by LTRs and the psi-sequence, which aids the packaging of the transgene into the virions. The assembled virus particles produced by the HEK293 cells are able to infect, and deliver the transgene to, target cells. The binding of virus particles to target cells is mediated by receptor-independent binding (Pizzato et al., 1999). This initial event is negatively influenced by strong electrostatic repulsion between the negatively charged cell and an approaching enveloped virus (Jensen et al., 2003, Swaney et al., 1997). The addition of positively-charged polycations, such as polybrene, reduces the repulsion forces and mediates the binding of the lentiviral particle to the cell surface resulting in a higher efficiency of transduction (Swaney et al., 1997, Le Doux et al., 2001). Once in the target cell, the transgene is imported into the nucleus and stably integrated into the host genome via the LTRs.

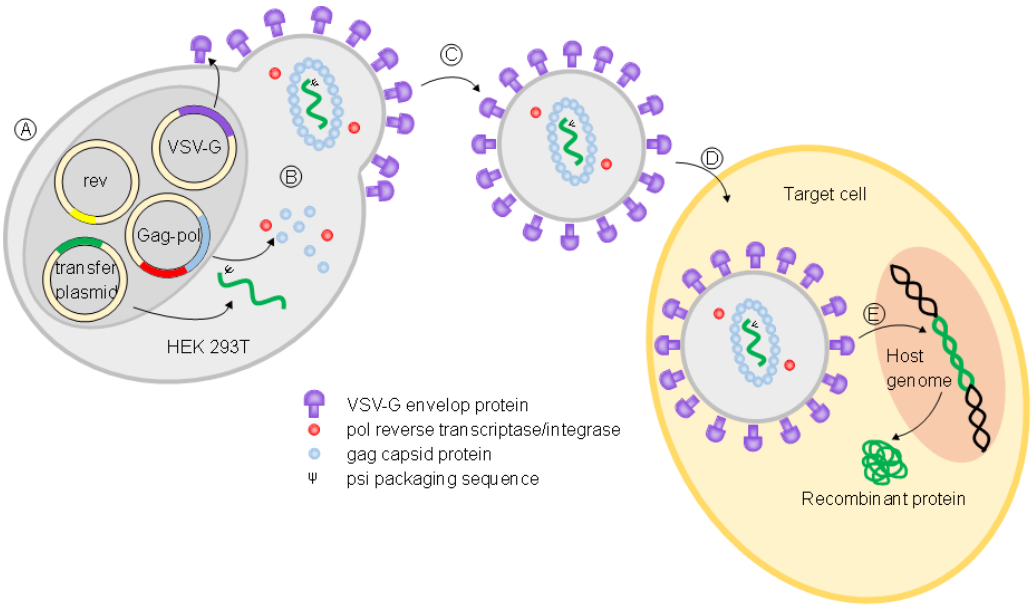


Figure 1-6: Lentiviral packaging and transduction. (A) The packaging cells are co-transfected with the four lentiviral plasmids (packaging plasmids, envelope plasmid and transfer plasmid). **(B)** This is followed by the assembly of the produced viral proteins to a virus particle. **(C)** The virion is then released into the cells supernatant. **(D)** The virus particles binds to the target cell by receptor-independent binding and infiltrates the cell. **(E)** The transgene is imported into the nucleus and stably integrated into the host genome mediated by the LTRs.

1.5 Aims

Brain metastases are the most frequently occurring intracranial neoplasms in adults (Eichler and Loeffler, 2007). However, treatment options for brain metastases are strongly limited by the poor access of drugs into the brain (Lockman et al., 2010). Therefore, novel treatments need to include strategies to overcome delivery limitations imposed by the BBB. Interestingly, the homing of microglia/macrophages to brain metastases has been demonstrated by various studies (He et al., 2006, Davoust et al., 2008, Lorger and Felding-Habermann, 2010). Moreover, myeloid precursor cells derived from bone marrow (bm) have been shown to enter the brain and differentiate into perivascular macrophages and occasionally microglia (Hickey and Kimura, 1988; Simard and Rivest, 2004). Furthermore, the bm-derived myeloid precursor cells are preferentially recruited to sites of neuronal degeneration (Priller et al., 2001). Based on these findings it can be concluded that the progeny of HSCs would be ideal cellular delivery vehicles for therapeutic agents to target brain metastases, as these can cross the BBB and infiltrate tumour lesions. Therefore, the overall aim of this thesis is to

develop a HSC-based therapy to treat brain metastases using genetically engineered HSCs to express a therapeutic agent.

Aim 1: Characterisation of brain metastases-infiltrating cells and their potential as therapeutic vehicles

To show that HSCs and their progeny can be exploited as cellular delivery vehicles into brain metastases, the goal was to investigate whether the myeloid brain tumour-infiltrating cells are of bm origin in experimental brain metastases models. The delivery of a transgene by the HSC progeny to brain metastases was also tested. Furthermore, to assess the translational potential of the cell therapy clinical brain metastases specimens were analysed for their proportion of infiltrating myeloid cells.

Aim 2: Development of an enzyme prodrug approach as a therapeutic option for HSC-based therapy to treat brain metastases

The most commonly used gene therapy approach for cancer is an enzyme prodrug system. The CD/5-FC therapy was developed as a therapeutic option of HSC-based cell therapy. The functionality of this system was assessed *in vitro* and *in vivo* following generation of the lentiviral transfer plasmid.

Aim 3: Developing a strategy for improved specificity of HSC-based cell therapy targeting brain metastases

The progeny of systemically administered HSCs can infiltrate different tissues. Following administration of HSCs that had been genetically engineered to express a therapeutic agent, an accumulation of agent would occur in brain metastases as well as in other tissues, and therefore potentially lead to systemic toxicities. A promoter that allows for specific expression of the therapeutic agent in brain metastases-infiltrating myeloid cells could circumvent this problem. Therefore, promoters that are specifically active in brain metastases-infiltrating myeloid cells were identified. Following validation of the activity of these promoters in murine and human brain metastases-infiltrating myeloid cells, the first steps towards promoter length optimisation for future application in the HSC-based cell therapy were performed

2. Materials and methods

2.1 Mammalian cell culture methods

2.1.1 Cell lines and cell culture

EO771 cells are medullary breast adenocarcinoma cells originally isolated from a spontaneous tumour from C57BL/6 mouse (Sugiura and Stock, 1952) and adapted for anti-cancer drug testing by Sirotnak et al. (1984). These cells were grown in Roswell Park Memorial Institute (RPMI)-1640 (Sigma) supplemented with 20% foetal bovine serum (FBS) (LabTech), 1% Glutamine (HyClone), 1% non-essential amino acids (Gibco), and 1% sodium pyruvate (Gibco).

PyMT cell line was derived from mouse mammary tumour virus (MMTV)-PyMT mouse model (Guy et al., 1992) and kindly provided by Professor Wolfram Ruf from The Scripps Research Institute, La Jolla, California. The cells were cultured in L-15 (Sigma), 10% FBS, 1% Glutamine, 10 µg/mL Insulin (Sigma).

The murine monocyte-macrophage cell line RAW 264.7 (American Type Culture Collection (ATCC)) used in this study was established from ascites of a tumour induced in a BALB/c male mouse by intraperitoneal (ip) injection of Abelson leukaemia virus (A-MuLV) (Raschke et al., 1978). This adherent cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), 10% FBS and 1% Glutamine.

HEK293 cells (ATCC) were grown in DMEM, 10% FBS and 1% Glutamine. This cell line has been derived from transformed human embryonic kidney cells by exposure to sheared fragments of adenovirus type 5 DNA (Graham et al., 1977).

All cell lines were grown as monolayers in 25 cm^2, 75 cm^2 or 150 cm^2 ventilated flasks (Corning) at 37 °C and 5% CO_2 in a humidified atmosphere. Medium was changed every other day and at 70-90% confluency cells were sub-cultured using 0.25% trypsin (HyClone) treatment for 2-5 min, followed by addition to a new flask with fresh medium.

2.1.2 Generation of lentiviral virus stocks

To transduce cells with lentiviral expression constructs lentiviral virus stocks were first produced. To this end, $2x10^6$ HEK293 cells were seeded in a 10 cm plate in 10 mL medium. The next day cells were transfected with the following reaction mixture:

Table 2-1: Transfection reaction mixture

Reagent	Amount for 1 plate
0.1xTE	450 µL minus plasmids
Gag-pol plasmid	6.5 µg
VSV-G plasmid	3.5 µg
Prsv-rev plasmid	2.5 µg
Transfer plasmid	10 µg
$CaCl_2$	50 µL
2xHBS	500 µL

2xHBS (281 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4) was added while the reaction mixture was vortexed. This mixture was added to the cells drop-wise and the plate was placed into 5% CO_2-incubator (Sanyo) overnight. The next morning medium was changed (5 mL per 10 cm plate). The virus-containing medium was collected 24 and 48 hours later, filtered through 0.45 µm pore filters and stored in aliquots at -80 °C.

Table 2-2: Lentiviral vectors used in this study

Name	Reference	Name	Reference
pFUGW	Lois et al. (2002)	pTREsCDHA	Designed for this study (see Appendix C)
pFUW-sCD	Designed for this study (see Appendix C)	pEZX	Purchased from GeneCopoeia
pFUW-sCDHA	Designed for this study (see Appendix C)	pEZX Dab2-mCherry	
pTREAutoR3	Markusic et al. (2005) Kindly provided by Professor André Lieber	pEZX MMP14-mCherry	
pTREsCD	Designed for this study (see Appendix C)	pEZX Spp1-mCherry	

2.1.3 Virus concentration

To concentrate generated lentiviral virus, the collected and filtered virus-containing medium (100-300 mL) was spun at 18,000 rpm at 4 °C for 2.5 h using the high speed centrifuge Avanti J-25 (Beckman Coulter) with the JA-18 rotor. The supernatant was carefully removed and the virus pellet was incubated on ice with either 100 µL phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA) or StemSpan SFEM (Stemcell Technologies) medium to loosen it. The loosened pellet was re-suspended and stored in aliquots at -80 °C.

2.1.4 Determination of virus titre

To determine the titre of the produced and concentrated virus stocks, one of the following two assays was performed.

2.1.4.1 Reverse transcriptase assay

The Reverse transcriptase (RT) assay (Roche) was used to quantitatively determine the RT activity of an aliquot of the virus stock. A virus stock with a known concentration was used as the standard for correlation. The standard virus stock used in this case was a pFUGW virus, whose titre was determined by flow cytometry analysis of HEK293 cells transduced with different amounts of the virus stock. Cells that were positively transduced with pFUGW virus were green fluorescent. The RT assay was performed according to manufacturer's instructions. The absorbance of the samples was determined using the plate reader Multiskan EX (Thermo Scientific) with Ascent software at an absorbance of 405 nm, and the readings were analysed using Microsoft Excel.

2.1.4.2 Provirus integration assay

The Lenti-X provirus quantitation kit (Clontech) was used to determine the number of integrated provirus copies in lentiviral transduced cells. This assay uses quantitative polymerase chain reaction (qPCR) with SYBR green chemistry to assess viral integration into genomic DNA by using primers that specifically bind to proviral insertion junctions.

The genomic DNA was extracted from $1x10^6$ transduced cells using the NucleoSpin Tissue kit (Machery&Nagel) 72 h after transduction. A calibrated provirus control template, supplied with the kit, was subjected to qPCR amplification alongside the samples and used as a control standard curve for analysis later on. The qPCR was performed as described in 2.2.1.3. The analysis of the qPCR results was performed according to the manufacturer's specification using Microsoft Excel.

2.1.5 Lentiviral transduction of cells

To transduce cells with lentiviral expression constructs, $4x10^5$ cancer cells were seeded in one well of a 6-well plate. 1 mL virus-containing media mixed with 8 µg polybrene (Sigma) was added and the plate incubated overnight. The next day the medium was

changed and protein expression analysis in transduced cells was performed after 48 hours or later.

In order to transduce mouse ScaI^{+} HSCs, 24-well plates were coated with 20 µg/mL retronectin (Clontech) in PBS at 4 °C overnight. After this solution was removed, the wells were incubated with a 2% BSA in PBS (w/v) solution at room temperature for 30 min and washed once with PBS. Then 2x10^{6} HSCs were plated into each well in a total volume of 0.5 mL StemSpan SFEM medium supplemented with murine stem cell factor (SCF) (100 ng/mL), murine thrombopoietin (TPO) (10 ng/mL), murine fms-related tyrosine kinase 3 ligand (FLT-3) (100 ng/mL) and murine IL-3 (20 ng/mL) (all growth factors and cytokines from Preprotech). To prepare the virus an appropriate amount of concentrated virus (multiplicity of infection ≥ 50) was added to HSC medium with 8 µg polybrene per mL medium and well mixed. The virus containing medium was added to the cells and left for an overnight incubation. The next morning the transduced cells were washed with PBS twice and then prepared for intravenous (iv) injection to complete the BMT.

2.2 Molecular biology and cloning

2.2.1 Polymerase chain reaction (PCR)

2.2.1.1 Two step PCR

In this study, a two-step PCR was used, which is a modification of PCR (Saiki et al., 1985), to introduce new short sequences additionally to the initial template DNA. This method was used to generate the DNA for the 5-FC converting enzyme cytosine deaminase (CD), using plasmid pORFCodA (InvivoGen) as a template and to attach a signal peptide sequence (tissue plasminogen activator (tPA)-signal) to its 5`end and a tag (HA-tag) to its 3`end. To ensure accuracy, a proof-reading polymerase, the Pfu Ultra HF polymerase (Agilent), was used to generate sCD (secretable CD). Oligonucleotide primers were ordered from Sigma-Aldrich (see

Table 2-3 for sequence) and dissolved in dH_2O to a 10 µM concentration. The PCR was performed using the Thermal Cycler Veriti (Applied Biosystems). Programmes used in this study as well as the reaction mixture composition are displayed in Table 2-4. PCR products were analysed by agarose gel electrophoresis.

Table 2-3: Primers used for two step PCR. Colour coding: **NdeI**, **CD**, **tPA signal**, **HA-tag**. Tm = annealing temperature (according to Sigma Aldrich), nt = nucleotides.

Primer name	Primer sequence	Tm (°C)	Primer length (nt)
sCD fwd	5'-AAGCAATCATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCA GTCTTCGTTTCGCCCAGCCAGGAAATCCATGCCCGATTCAGAAGAATGAGCAATAACG-3'	66.3	117
sCD rev	5'-GTAACCCAGTCGTTCAACGTTTG-3'	53	23
sCDHA rev	5'- TCAAGCGTAGTCTGGGACGTCGTATGGGTAAGATCCAGAAGCGTAGTCTGGGACGTC GTATGGGTAACGTTTGTAATCGATGGC -3'	92.2	84
tPA_NdeI fwd	5'- TGTACCATGCATATGGAAGCAATCATGGATG-3'	75.4	31
sCDHA 2 rev	5'- CTATCAAGCGTAATCTGGAACATCGTATGGGTAACGTTTGTAATCGATGGC-3'	82.4	51

Table 2-4: PCR programme and reaction mixture for two step PCRs.

PCR reaction mixture			PCR programme								
			sCD (1388 bp)			sCDHA (1451 bp)			sCDHA2 (1431 bp)		
DNA template	100ng		Initial denaturation	95 °C	2 min	Initial denaturation	95 °C	2 min	Initial denaturation	95 °C	2 min
Primer fwd	1 µM	10x	Denaturation	95 °C	30 sec	Denaturation	95 °C	30 sec	Denaturation	95 °C	30 sec
Primer rev	1 µM	10x	Annealing	34°C	30 sec	Annealing	34 °C	30 sec	Annealing	41 °C	30 sec
10x Pfu pol buffer	5 µL	10x	Elongation	72 °C	1 min	Elongation	72 °C	1 min	Elongation	72 °C	1 min
dNTPs (20 mM)	1 µL	20x	Denaturation	95 °C	30 sec	Denaturation	95 °C	30 sec	Denaturation	95 °C	30 sec
$MgSO_4$ (50 mM)	1 µL	20x	Annealing	72 °C	30 sec	Annealing	72 °C	30 sec	Annealing	67 °C	30 sec
ddH_2O	up to 50 µL	20x	Elongation	72 °C	1 min	Elongation	72 °C	1 min	Elongation	72 °C	1 min
Pfu polymerase	1 µL		End- Elongation	72 °C	10 min	End- Elongation	72 °C	10 min	End- Elongation	72 °C	10 min
	50 µL		Hold	4 °C	∞	Hold	4 °C	∞	Hold	4 °C	∞

2.2.1.2 Semi-quantitative reverse transcriptase PCR (RT-PCR)

This method was performed to detect the level of expression of mRNA in a semi-quantitative way and thus to compare levels of transcripts in different samples. As comparison of the intensities of PCR product bands can only be performed during the logarithmic amplification phase, PCR cycle numbers for each individual gene specific primer set were determined first. The expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalise gene expression in each sample. Gene-specific primer set (Sigma Aldrich) sequences, annealing temperatures and the number of cycles are given in Table2-5. All PCR reactions were performed with Pfu Ultra HF DNA polymerase (Agilent) in a reaction volume of 12 µL using the same amount of cDNA for each reaction. The PCR programme was as follows: 95 °C for 2 min, cycle number x (95 °C for 2 min, annealing temperature for 30 sec, and 72 °C for 30 sec) and 72 °C for 10 min. Products were separated on a 2% agarose gel containing ethidium bromide. Images of PCR products were obtained and analysed using ImageJ software.

Table 2-5: Semiquantitative RT-PCR primer sequences

Gene	Forward primer	Reverse primer	Tm (°C)	Cycles	cDNA size (bp)
Spp1	5'-GCTTGGCTTATGGACTGAGGTC-3'	5'-CCTTAGACTCACCGCTCTTCATG-3'	53	30	114
MMP-14	5'-GATGGACACAGAGAACTTCGTG-3'	5'-CGAGAGGTAGTTCTGGGTTGAG-3'	53	30	117
Trem2	5'-CTACCAGTGTCAGAGTCTCCGA -3'	5'-CCTCGAAACTCGATGACTCCTC -3'	53	30	134
Dab2	5'-CTCTTCAAAGGCAATGCTCCTGC-3'	5'-TATGGCTCCTGGGACCACAGTT-3'	55	30	134
Emp1	5'-TCCCTGTCCTACGGCAATGAAG-3'	5'- CTGGAACACGAAGACCACAAGG-3'	60	35	169
Arg1	5'-CATTGGCTTGCGAGACGTAGAC-3'	5'-GCTGAAGGTCTCTTCCATCACC-3'	53	30	124
Ccl7	5'-CAGAAGGATCACCAGTAGTCGG-3'	5'- ATAGCCTCCTCGACCCACTTCT-3'	55	30	108
Cxcl10	5'-ATCATCCCTGCGAGCCTATCCT-3'	5'-GACCTTTTTTGGCTAAACGCTTTC-3'	55	32	134
Cxcl16	5'-GCAGGGTACTTTGGATCACATCC-3'	5'-AGTTCACGGACCCACTGGTCTT-3'	57	32	126
Ccr5	5'-GTCTACTTTCTCTTCTGGACTCC-3'	5'-CCAAGAGTCTCTGTTGCCTGCA-3'	54	35	131
GAPDH	5'-GCACAGTCAAGGCCGAGAAT-3'	5'-GCCTTCTCCATGGTGGTGAA-3'	54	40	151

2.2.1.3 Quantitative real time PCR (qPCR)

Quantification of RNA expression using synthesised cDNA was performed with two different fluorescent dye systems in this study.

In the context of the Lenti-X provirus quantitation kit (Clontech), SYBR green was used as a fluorescent dye to determine the number of integrated provirus copies in lentiviral transduced cells. A calibrated provirus control template was subjected to qPCR amplification alongside the samples and used as a control standard curve for later analysis. This kit, using Lenti-X provirus forward and reverse primers, specifically amplified proviral insertion junctions. PCR programme and reaction mixture are displayed in the table below.

Table 2-6: SYBR Green qPCR reaction mixture and thermal cycling conditions.

PCR reaction mixture		**Thermal cycling conditions**		
cDNA (100 ng/µL)	10 µL	**Initial Denaturation (1cycle)**	95 °C	30 sec
PCR-grade H_2O	6.8 µL	**qPCR (40 cycles)**	95 °C	34 sec
Lenti-X provirus primer fwd	0.4 µL		60°C	31 sec
Lenti-X provirus primer rev	0.4 µL	**Dissociation curve (1cycle)**	95 °C	15 sec
ROX Reference Dye LMP	0.4 µL		60 °C	1 min
SYBR Advantage qPCR Premix (2x)	10 µL		95 °C	15 sec
Volume/well	**20 µL**			

To determine sCD expression levels in leukocytes from mice that received BMT with sCD transduced HSCs, a TaqMan gene expression assay (Life technologies) was performed. Expression of the house keeping gene GAPDH was used as a normalisation control for each sample. Thermal cycling conditions as well as PCR reaction mixture for TaqMan qPCRs are given in Table 2-7.

Table 2-7: TaqMan qPCR reaction and thermal cycling conditions.

PCR reaction mixture	
cDNA (25 ng)	3 µL
PCR-grade H_2O	3.75 µL
Primer (sCD/GAPDH) probe	0.75 µL
TaqMan buffer	7.5 µL
Volume/well	**15 µL**

Thermal cycling conditions		
Hold	50 °C	2 min
Hold	95 °C	10 min
Cycle (40 cycles)	95 °C	15 sec
	60 °C	1 min

All samples were analysed in triplicates using the thermal cycler ABI 7500 Real-Time PCR System (Applied Biosystems) and the relative expression was calculated by the comparative Ct method.

2.2.2 Purification of PCR products

Generated PCR products were purified from PCR reaction mixture using the QIAquick PCR Purification Kit (QIAGEN) according to the kit protocol.

2.2.3 Blunt end filling of PCR products with Klenow fragment

The 5´-3´polymerase activity of the Klenow fragment (Fermentas) was used to generate blunt ends on PCR products generated by Pfu Ultra HF polymerase. The reaction mixture was incubated at room temperature for 20 min and inactivated at 70 °C for 10 min.

Table 2-8: Klenow fragment reaction mixture

Reaction components	Quantity
DNA	3 µg
dNTPs (10 mM)	0.4 µL
10x Klenow buffer	4 µL
Klenow enzyme	1 µL
ddH_2O	up to 40 µL
Total volume:	40 µL

2.2.4 Phosphorylation of PCR products

DNA fragments generated by PCR lack 5`-P and need to be phosphorylated prior to ligation. In this study, T4 Polynucleotide Kinase (T4 PNK) (Fermentas) was used to catalyse the transfer of the γ-phosphate from ATP to the 5'-OH group of single- and double-stranded DNAs and RNAs or oligonucleotides.

The reaction mixture (see Table 2-9) was incubated at 37 °C for 20 min and inactivated at 70 °C for 10 min.

Table 2-9: T4 PNK reaction mixture

Reaction components	**Quantity**
DNA (purification kit)	30 µL
ATP (100 mM)	4 µL
10x T4 PNK buffer	4 µL
T4 PNK enzyme	2 µL
ddH_2O	up to 40 µL
Total volume:	40 µL

2.2.5 Restriction digest

Restriction enzymes recognise a restriction site (specific palindromic nucleotide sequence) at which they cut a double-stranded DNA to produce sticky or blunt ends. Various Fast Digest (FD) restriction endonucleases were used in this study, i.e. HpaI, BamHI, NdeI, PstI, AgeI (Thermo Scientific), either for cloning or for screening purposes. Reactions were set up according to the manufacturer's instructions (see table below). Screening digests were performed for 15 min at 37 °C, and digests for cloning for 1hr at 37 °C. Subsequently restriction digests were analysed by agarose gel electrophoresis.

Table 2-10: Restriction digest reaction mixture

Reaction components	Quantity
DNA	1 to 2 µg
10x FD restriction digest buffer	2 µL
FD restriction enzyme	1 µL
ddH_2O	up to 20 µL
Total volume:	20 µL

2.2.6 Vector DNA de-phosphorylation

To prevent auto-ligation of the vector backbone after blunt end cutting, a de-phosphorylation step was performed (see Table 2-11).

The reaction mixture was incubated at 37 °C for 30 min. After addition of an additional 1 µL calf intestine alkaline phosphatase (CIAP) (Fisher Scientific) a second incubation was performed at 37 °C for 30 min. Subsequently, an inactivation step was performed at 68 °C for 10 min.

Table 2-11: Dephosphorylation reaction mixture

Reaction components	Quantity
Restriction digest reaction mixture	20 µL
10x CIAP buffer	3 µL
CIAP	1 µL
ddH_2O	6 µL
Total volume:	30 µL

2.2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a range of 0.8-2% (w/v) agarose gels (containing 0.5 µg/mL ethidium bromide (Alfa Aesar)), depending on the DNA sizes expected. Gels were made with and were run in 1x TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM ethylenediaminetetraacetic acid (EDTA) pH 8.3). Samples were mixed with 6x DNA Loading Dye (Thermo Scientific), loaded and subjected to an electric current of 100 V. Electrophoresis was performed for 30-60 min depending on the size of

the expected bands. Products were visualised using ultra violet (UV) light in a GelDoc XR system (BioRad).

2.2.8 DNA gel extraction

To purify and isolate linearised DNA fragments from agarose gel, the Zymoclean Gel Recovery Kit (Zymo Research) was used according to manufacturer's instructions.

2.2.9 Ligation of DNA fragments

To create recombinant DNA, ligases are used to join linear DNA fragments together with covalent bonds by generation of a phosphodiester bond between the 3'-hydroxyl of one nucleotide and the 5'-phosphate of another. In this study, Rapid DNA Ligation Kit (Roche) was used to ligate PCR products into vector.

The reaction mixture (see Table 2-12) was incubated at room temperature for 15 min and subsequently transformed into *E.coli* cells (see 2.2.10).

Table 2-12: Ligation reaction mixture

Reaction components	**Quantity**
Backbone : insert ratio	1:3 / 1:5 / 1:10
5 x ligase buffer	2 µl
2 x ligase buffer	5 µl
ligase	0.5 µl
ddH_2O	add to 10 µl
Total volume:	10 µl

2.2.10 Heat shock transformation of plasmid DNA into *E.coli*

To insert a foreign plasmid or ligation product into chemically competent bacteria, the heat shock method is widely used. One Shot Top10 Chemically Competent *E.coli* (Invitrogen) cells were thawed on ice for 10 min. After adding either 5 µL of a ligation reaction mixture or 0.1 µg of plasmid DNA to a volume of 50 µL competent cells, the mixture was incubated on ice for 20 min and a 90 sec heat shock followed at 42 °C. The

tube was placed immediately back on ice for 5 min and after the addition of 500 µL lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl), the cells were put in a bacterial shaker at 37 °C for 45 min. The cells were then centrifuged at 1,000 rpm for 3 min and the re-suspended pellet was spread on an LB plate, containing ampicillin at 100 µg/mL. After overnight cultivation at 37 or 30 °C, bacterial colonies were picked for screening purposes.

2.2.11 Isolation of plasmid DNA

For the purpose of purifying plasmid DNA from *E.coli* cultures, commercial kits (QIAprep Spin Miniprep Kit, QIAGEN Plasmid Maxi Kit, Life Technologies HiPure Plasmid Midiprep Kit) were used according to the appropriate kit protocol.

The purified DNA was quantified and analysed by NanoDrop (Thermo-Scientific) and agarose gel electrophoresis, respectively.

2.2.12 DNA sequencing

To verify successful cloning, the plasmid DNA was sequenced. Cloned constructs were sent to either Eurofins MWG Operon or Source Bioscience to be sequenced by modified chain termination method (Sanger et al., 1977). The obtained sequences were then analysed by appropriate software (ApE A plasmid editor).

2.2.13 Isolation of genomic DNA (gDNA)

Genomic DNA was isolated using the NucleoSpin tissue genomic DNA purification kit (Macherey-Nagel). The procedure was performed according to manufacturer's instructions. The concentration of gDNA was determined by absorbance at 260 nm on a NanoDrop.

2.2.14 Isolation of RNA

To isolate total RNA from samples to use for downstream applications, such as qPCR, RT-PCR as well as GEX, the RNAqueous-Micro Kit (Life technologies) according to the

manufacturer's protocol was used. The amount of total RNA isolated was quantified using a NanoDrop.

2.2.15 Synthesis of copy DNA (cDNA)

To perform semiquantitative RT-PCR or qPCR, isolated RNA was transcribed into cDNA using Superscript (SS) III First strand synthesis kit (Thermo Fisher Scientific) with the Thermal Cycler Veriti (Applied Biosystems). In brief, reaction mixture 1 (see Table 2-13) was incubated at 65 °C for 5 min. After 1 min incubation on ice reaction mixture 2 was added, followed by an incubation at 50 °C for 60 min and inactivation step at 70 °C for 15 min.

Table 2-13: cDNA synthesis reaction mixture

	Reaction components	**Quantity**
Mix 1	RNA (plus H_2O to 11 µL total)	0.5 µg
	oligo $(dT)_{20}$ primer	1 µL
	10 mM dNTPs (nucleoside triphosphate)	1 µL
Mix 2	5x FirstStrand synthesis buffer	4 µL
	0.1 M DTT (dithiothreitol)	1 µL
	RNase OUT	1 µL
	SS III reverse transcriptase	1 µL
	Total volume:	20 µL

2.3 *In vivo* methods

2.3.1 Mouse strains

Female C57BL/6 mice were purchased from St James's Biomedical Service (SBS) or Charles River Laboratories (CRL). Transgenic C57BL/6 UBC-GFP mice (Schaefer et al., 2001) from Jackson laboratory were also used in this study. These animals express enhanced green fluorescent protein (GFP) under the control of the human ubiquitin C (UbC) promoter in all tissues.

All mice were maintained in the SBS animal care facilities at the University of Leeds. Animal experiments were performed under approved Home Office licence in accordance with the Animals (Scientific Procedures) Act 1986 and the UK National Cancer Research Institute Guidelines for the welfare of animals (Workman et al., 2010).

2.3.2 Whole body mouse irradiation

For bone marrow transplantation (BMT) it was first necessary to ablate the mouse haematopoietic system. Therefore, a whole body irradiation was performed. To this end, caged mice were placed into an RS-2000 irradiator (Rad Source Technologies) and irradiated with a single dose of 845 Rad (=8.45 Gy). Subsequently, the mice started receiving Baytril (Bayer) in drinking water (0.2 mL of 2.5% stock solution per 100 mL water) until the end of the experiment.

2.3.3 Magnetic cell separation (MACS)

To perform a BMT, ScaI$^+$ HSCs need to be isolated from extracted bone marrow (bm) and then injected into irradiated mice. After culling of mice, femur and tibia were isolated. The bone marrow was flushed out with incubation buffer (PBS/2 mM EDTA/0.5% BSA) using a needle and syringe. Bone marrow cells were passed through a 70 µm pore nylon strainer to reduce the possibility of column clogging. ScaI$^+$ HSCs were isolated using MACS separation columns (Miltenyi) with the Anti-Sca-I MicroBead Kit (FITC) (Miltenyi) according to the manufacturer`s protocol. The isolated ScaI$^+$ cells were either transduced with a lentiviral expression construct overnight or directly injected iv at $5x10^5$ cells per mouse in 100 µL PBS.

A MACS procedure was also performed for the isolation of CD11b$^+$ cells from bm and spleen samples using the CD11b MicroBeads, human and mouse kit (Miltenyi). Isolated RNA from these cells was subsequently used for gene expression profiling (see 2.4). Furthermore, the Myelin Removal Beads II kit (Miltenyi) was used to deplete myelin debris from single cell brain tumour samples prior to their use in flow cytometry experiments or in Western blotting.

2.3.4 Implantation of cancer cells into mouse brain

The intracranial implantation of cancer cells into mice was used as a model to analyse brain metastases in this study. After induction and maintenance of anaesthesia, mice were restrained in a specialised stereotactic apparatus (Stoelting). Their shaved heads were disinfected using Hibiscrub and 70% ethanol. Metacam and Baytril were injected at the beginning of the surgery. A cut into disinfected skin with a scissor in the direction of the nose was performed. The exposed underlying membrane was left to dry followed by the penetration of the skull at specific coordinates (2 mm right from the midline, 2 mm anterior from bregma) using the Ideal Micro Drill (Roboz Surgical Store). Then a needle containing the cell suspension ($1x10^5$ in a total volume of 2 µL) was placed exactly over the hole in the skull, followed by its immersion into the brain at a depth of 4 mm and a needle retraction of 1 mm to create a little hole in the striatum. The cell suspension was injected in 1 µL aliquots, leaving 1 min in-between to allow for absorption of the injected fluid. The needle was then slowly withdrawn by 1 mm every 30 sec. The hole was immediately sealed with bone wax (Harvard Apparatus). The skin was closed with Vetbond Tissue Adhesive (3M) and an antibiotic gel was applied onto the wound. The mice were placed into a recovery chamber for at least 30 min to recover from anaesthesia.

2.3.5 Blood draw from the tail vein

To determine whether the bone marrow of mice that had undergone a BMT was successfully reconstituted, the blood was analysed. To this end mice were put in a warming chamber at 37 °C for 20 min to maximise vasodilation. The tail of a mouse was pinched with a 25 G needle and blood droplets were collected in a tube containing 1 mL red blood cell lysis buffer (150 mM NH_4Cl/ 10 mM $NaHCO_3$/1 mM EDTA) to prevent coagulation. After a 2 min incubation at room temperature, the isolated cells were washed with incubation buffer twice and were ready to be used in further applications, i.e. flow cytometry analysis.

2.3.6 Bioluminescence imaging

This technology was used to study the biological process of brain tumour growth *in vivo* in a non-invasive way. The cancer cells used for intracranial implantation were lentivirally transduced to express Firefly Luciferase (FLuc). The mice were anaesthetised prior to ip injection of 1.2 mg D-Luciferin potassium salt (Regis Technologies; stock 15 mg/mL in

PBS). To ensure complete distribution of luciferin throughout the body, the imaging was performed 8 min after luciferin injection. The bioluminescent light emitted by the FLuc-Luciferin reaction was read by IVIS Spectrum (Perkin Elmer). The corresponding data was analysed using the LivingImage3.2 (Perkin Elmer) software.

2.3.7 Isolation of splenocytes

In this study, RNA isolated from $CD11b^+$ splenocytes was used for gene expression profiling to compare gene expression between $CD11b^+$ cells from brain, bm and spleen. To isolate splenocytes, mice were terminally perfused and their spleens isolated and kept on ice. The splenocytes were gently squeezed out of the spleen and collected into incubation buffer. After a centrifugation step (400 g at 4 °C for 10 min) the cells were re-suspended and incubated in red blood cell lysis buffer at room temperature for 3-4 min. Thereafter, the splenocytes were washed three times with incubation buffer and were ready to use in subsequent applications, i.e. MACS or flow cytometry.

2.3.8 Dissociation of brain tumour tissue

Murine brain tumours need to be dissociated into a single cell suspension in order to isolate distinct cell populations, prepare cell lysates or to investigate the composition cell populations. Firstly, mice were perfused with 0.9% saline and the brain was isolated and its frontal part, containing the tumour tissue, placed on ice. Then 500 µL of collagenase solution (3 mg/mL collagenase A (Roche), 250 U/mL hyaluronidase (Sigma) in EMEM) was added and the tissue was chopped up with scalpels. The mixture was incubated at 37 °C for 10 to 20 min and dissociated by pipetting in-between. Cells were washed with incubation buffer and passed through a 70 µm nylon strainer to reduce the possibility of clogging. After myelin debris was removed using MACS (see 2.3.3), the single cell suspension was ready for use in subsequent applications.

2.4 Gene expression profiling (GEX)

Total RNA of bone marrow-derived myeloid cells ($CD11b^+GFP^+$) isolated from brain metastases (EO771 and PyMT model) and myeloid cells isolated from spleen and bone marrow were sent to Cambridge Genomic Services to perform whole genome gene expression studies using the Illumina BeadArray system. Samples were processed with

the TotalPrep RNA amplification kit (Ambion) and hybridised to the MouseRef-8v2 WG-GX beadchip (Illumina) following the direct hybridisation assay. Biological replicates used for GEX were as follows: n=4 for PyMT brain tumour samples and spleens of PyMT tumour-bearing mice, n=2 for EO771 brain tumours, naïve spleens, and naïve bone marrow, and n=1 for the bone marrow of PyMT brain tumour-bearing mice.

The bioinformatic analysis of the raw data was performed by Dr Alastair Droop (University of Leeds) using GenomeStudio version 2011.1 (Illumina), as well as R/Bioconductor for data processing and visualisation. To correct for background, he used the "normexp" algorithm (Ritchie et al., 2007), and normalisation between arrays was performed using A-value quantile normalisation (Yang and Thorne, 2003). Probes that were expressed in fewer than 3 arrays (using a detection p-value of < 0.05) were removed and the differential gene expression analysis between groups was performed using a linear model in LIMMA (FDR = 1%) (Smyth, 2004).

2.5 Protein expression analysis

2.5.1 Immunofluorescence (IF) analysis

2.5.1.1 Human samples

Breast cancer brain metastases tissue was obtained from the Leeds Multidisciplinary Research Tissue Bank. The study was approved by the Leeds (East) Research Ethics Committee. Obtained samples were separated into two to four equal sized parts depending on the sample size. One part was fixed in 4% Paraformaldehyde (PFA) (Sigma Aldrich) in PBS at 4 °C for 48 h and subsequently transferred into sucrose solution (25% sucrose (w/v)/ sodium phosphate (monobasic)/ 77 mM sodium phosphate (dibasic)) for 3 days. After the tissue was embedded in optimal cutting temperature compound (OCT) (VWR Chemicals), it was cut into 10 µm thick sections onto slides using CM-3050S cryostat (Leica) and stored at -20 °C. Another part of the sample was fixed in neutral buffered formalin (NBF) solution (37% formalin solution/ 4 g/L sodium phosphate (monobasic) / 6.5 g/L sodium phosphate (dibasic)) at room temperature for 48 h, transferred into 70% ethanol before it was embedded in paraffin. The embedded samples were cut into 5 µm thick sections onto Superfrost plus slides (Thermo Scientific) using microtome and stored at room temperature.

Donor-matched blood samples collected into EDTA-coated tubes were incubated with red blood cell lysis buffer (Biolegend) at room temperature for 4 min. After 2 washes with

HBSS the cell pellet was fixed with 500 µL NBF for 30 min. Following a centrifugation step at 300 g for 10 min the fixed cells were stored in 70% ethanol prior to paraffin embedding. The sample was cut into 5 µm thick sections and stored at room temperature.

2.5.1.2 Murine samples

To analyse murine brain tumour samples by IF the brains of tumour bearing mice were isolated after terminal perfusion with PBS. Splenocyte and bm cell pellets as well as whole brains were either PFA fixed OCT embedded or NBF fixed paraffin embedded (see section above). All embedded material was cut into 10 µm thick sections onto slides.

2.5.1.3 De-paraffinisation and antigen heat retrieval

Paraffin embedded material needed to be dewaxed to be used for IF staining. To this end slides were incubated with Xylene twice for 10 min. Then the sections were re-hydrated using a decreasing ethanol row (100%, 95%, 85%, 70%, 50% and 25% EtOH for 2 min each) and finally were washed with dH_2O twice for 5 min.

All antibodies used in this study for IF staining required antigen retrieval of the dewaxed sections. For this purpose a heat-induced epitope retrieval was performed by incubating the slides in 1 mM EDTA at 95-100 °C for 10 min. The slides were ready for staining after cooling to room temperature for approximately 45 min.

2.5.1.4 Immunofluorescence (IF) staining

To determine expression and localisation of proteins in tissue samples IF staining was performed. Slides were washed 3x in PBS for 5 min and each section was encircled using a hydrophobic PAP Pen. After an hour incubation with blocking buffer (PBS / 10% goat serum / 0.03% Triton-X-100), the primary antibody diluted in blocking buffer was added for either 2 h or overnight. The slides were washed 3x with PBS for 5 min, followed by an incubation with the secondary antibody diluted in blocking buffer. Then 4',6-diamidino-2-phenylindole (DAPI) (10 mg/ml stock; 1:5000 in PBS) was added for 10 min and washed off 3x with PBS for 5 min. Lastly, the slides were mounted with cover glasses (Menzel-Glaeser) using 80 µL Prolong Gold Antifade (Life Technologies) per slide.

Immunofluorescence signal was visualised using the fluorescence microscope ApoTome (Zeiss) and EVOS FL (Life Technologies) and the confocal microscope A1R (Nikon).

Table 2-14: Antibodies used for IF stainings

	Name	Company	Species	Dilution
Primary antibody	α-human **CD68**	DAKO	Mouse	1/100
	α-hemagglutinin (**HA**) tag	Covance	Mouse	1/100
	α-**pan-cytokeratin** (C11)	Santa Cruz	Mouse	1/100
	α-**GFP**	Chemicon	chicken	1/100
	α-**DAB2**	Bioss	Rabbit	1/100
	α-**Emp1**	Abbiotec	Rabbit	1/100
	α-**TREM2**	Bioss	Rabbit	1/100
	α-**MMP14**	GeneTex	Rabbit	1/100
	α-**SPP1**	GeneTex	Rabbit	1/100
Secondary antibody	α-mouse A488	Invitrogen	Goat	1/500
	α-mouse A594	Invitrogen	Goat	1/500
	α-rabbit A488	Invitrogen	Goat	1/500
	α-rabbit-TRITC	Jackson	Donkey	1/200
	α-chicken-FITC	Jackson	Donkey	1/200

2.5.2 Western blotting

2.5.2.1 Preparation of samples

In order to determine if cells express the protein of interest by Western blotting, cells are lysed first. Cell pellets were re-suspended in an appropriate amount of radioimmunoprecipitation assay (RIPA) lysis buffer solution (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.5 mM DTT, 1 mM Na_3VO_4, 50 mM NaF, 0.5% DOC, 0.1% sodium dodecyl sulfate (SDS), 25 mM β-Glycerophosphate, 1x cOmplete Mini (Roche)). After 20 min of incubation on ice, the suspension was centrifuged at 13,000 rpm at 4 °C for 15 min, the protein lysate was separated from cell residues and stored at -20 °C.

To verify secretion of the expressed protein sCD, the supernatant of cultured transduced cells was concentrated by ultrafiltration. The conditioned medium was spun at 2,000 g for 5 min to remove cell debris. The supernatant was transferred into an Amicon Ultra-

15ml (Merck Millipore) device with a molecular cut off weight of 30 kDa and spun at 4,000 g for 20 min. The concentrated media sample was stored at -20 °C.

2.5.2.2 Determination of protein concentration

The protein concentration of samples was determined by using the Pierce BCA (bicinchoninic acid) protein assay kit (Thermo Scientific) according to the manufacturer's protocol. Sample aliquots were diluted 1:10 with dH_2O prior to application. The plate reader Multiskan EX (Thermo Scientific) with Ascent software was used to measure absorbance at 562 nm and the readings were analysed using Microsoft Excel.

2.5.2.3 SDS-polyacrylamide gel electrophoresis

Protein separation to determine the relative abundance of a protein in a sample was performed by SDS-PAGE. The resolving and stacking gel solutions (see Table 2-15) were cast into a Bio-Rad device. The same amount of protein for each sample was mixed with 6x Laemmli sample buffer (300 mM Tris pH 6.8, 600 mM DTT, 12% SDS, 0.6% bromphenol blue, 60% glycerin) and denatured at 95 °C for 5 min. After filling the electrophoresis chamber with electrophoresis buffer solution (25 mM Tris Base, 192 mM glycine, 0.1% SDS(w/v)), the prepared probes and SeeBlue Plus2 Prestained Standard (Invitrogen) were loaded into the wells. An electric field of 100 V was applied to separate proteins according to their molecular mass.

Table 2-15: Composition of resolving and stacking gel

	Resolving gel 12.5 %	**Stacking gel 4%**
ddH_2O	1.375 mL	1.423 mL
1 M Tris	1.875 mL (pH 8.8)	0.25 mL (pH 6.8)
40% Protogel (National Diagnostics)	1.67 mL	0.267 mL
SDS (20%)	25 µL	10 µL
APS (10%) (Sigma)	50 µL	50 µL
TEMED (Sigma)	3 µL	3 µL

2.5.2.4 Western blot

Following SDS-PAGE, separated proteins were transferred from the electrophoresis gel to a nitrocellulose membrane and target proteins were then detected using specific antibodies.

Separated proteins were transferred using a wet blot technique. The blot chamber was filled with transfer buffer (25 mM Tris Base, 192 mM glycine, 0.1% SDS (w/v), 20% methanol (v/v)) and proteins were transferred to a membrane at 0.3 A and 100 V at room temperature for 2 h. Subsequently, the membrane was blocked with milk protein solution (1 % milk powder in TBS-T) for 1 hour before being incubated with a primary antibody solution at room temperature overnight. Unbound primary antibodies were removed by washing the membrane with TBS-T (50 mM Tris pH 7.5/ 150 mM NaCl/ 1% Triton-X-100) 3x for 10 min followed by a secondary antibody incubation for 1 hour. Unbound antibodies were washed off with TBS-T 3x for 10 min. The Pierce ECL western blotting substrate 1 and 2 (Thermo Scientific) were mixed 1:1, added to the membrane and incubated for 1 min. Emitted light was transferred onto an Amersham Hyperfilm ECL (GE Healthcare), which was developed by the X-ray developer SRX-101A (Konica).

Table 2-16: Antibodies used for WB

	Name	Company	Species	Dilution
Primary antibody	α-hemagglutinin (**HA**) tag	Covance	mouse	1/10000
	α-alpha-**Tubulin**	GeneTex	mouse	1/10000
Secondary antibody	α-rabbit-HRP	Invitrogen	goat	1/50000
	α-mouse-HRP	Invitrogen	goat	1/50000

2.5.3 Flow cytometry

In this study, flow cytometry was used for various different analyses, e.g. to determine the reconstitution efficiency of bone marrow transplantations, to identify brain tumour infiltrating leukocyte populations, and to confirm overexpression of genes in transduced cells.

2.5.3.1 Sample preparation

Single cell suspensions were washed with incubation buffer once. Prior to incubation with murine-specific antibodies (produced in rat), cells were blocked with 10% rat serum, and cells undergoing incubation with human-specific antibodies (produced in mouse) were blocked with 10% mouse serum for 10 min. Samples were incubated with antibodies on ice for 30-45 min and then washed again in buffer before analysing using the flow cytometer BD LSRII (BD Bioscience). The data analysis was performed with BD FACSDiva software, a flow cytometry data analysis software.

Table 2-17: Antibodies used for flow cytometry

	Name	Company	Species	Isotype	Dilution
Primary antibody	α-hemagglutinin (**HA**) tag	Covance	mouse	IgG1	0.25 uL/1x10^5 cells
	APC α-mouse **Ly-6G**	Biolegend	rat	IgG2a,κ	0.06 µg/1x10^6 cells
	PE α-mouse Ly-6G (**Gr-1**)	eBioscience	rat	IgG2b,κ	0.03 µg/1x10^6 cells
	PE α-mouse **Ly-6C**	BD Bioscience	rat	IgM, κ	0.50 µg/1x10^6 cells
	APC α-mouse **CD45**	Biolegend	rat	IgG2b,κ	0.25 µg/1x10^6 cells
	PE α-mouse **CD45**	Biolegend	rat	IgG2b,κ	0.25 µg/1x10^6 cells
	APC α-mouse **CD11b**	Biolegend	rat	IgG2b,κ	0.25 µg/1x10^6 cells
	V450 α-mouse **CD11b**	BD Biosciences	rat	IgG2b,κ	0.50 µg/1x10^6 cells
	APC α-mouse **F4/80**	AbD Serotec	rat	IgG2b	0.05 µg/1x10^6 cells
	PE α-mouse **CD19**	Biolegend	rat	IgG2a,κ	0.25 µg/1x10^6 cells
	APC α-**CD3e**	eBioscience	rat	IgG2b,κ	0.25 µg/1x10^6 cells
	V450 α-**B220**	eBioscience	rat	IgG2a,κ	0.25 µg/1x10^6 cells

	Name	Company	Dilution
Isotype control	APC rat (IgG2b,κ)	Biolegend	Same amount as used for staining
	APC rat IgG2a,κ	eBioscience	
	PE rat IgG2b,κ	eBioscience	
	V450 rat IgG2b,κ	eBioscience	
	PE rat IgM	BD Bioscience	

2.5.3.2 Intracellular staining

Intracellular staining of cells was performed when the epitope of interest was not displayed on the cell surface. Cells were fixed with 4% PFA for 10 min and washed with incubation buffer. The samples were then incubated with incubation buffer/0.1% Triton-X-100 for 10 min to increase the permeability of the cell's surface. The cells were then stained using the same protocol as described in 2.5.3.1.

2.5.3.3 Fluorescence-activated cell sorting (FACS)

FACS enables the isolation of a specific cell population from a heterogeneous cell mixture. In this study, it was used to isolate $CD11b^+/GFP^+$ cells from dissociated brain tumour tissue. Cells were incubated with CD11b allophycocyanin (APC) antibody (BD Biosciences) 0.2 µg/1x10^6 cells in incubation buffer for 45 min. After a wash step with incubation buffer, cells were sorted using MoFlo Legacy (Beckman Coulter) and collected into pre-chilled FACS tubes containing 1.5 mL PBS with 2% FBS. Collected cells were spun down at 400 g, 4 °C for 10 min, counted, aliquoted in screw top vials,

and washed with ice-cold PBS. Vials containing cell pellets were placed on dry ice for 5 min and frozen down at -80 °C.

3. Characterisation of brain metastases-infiltrating cells and their potential as therapeutic vehicles

3.1 Introduction

In recent decades the occurrence of brain metastases originating from lung cancer, breast cancer and melanoma has increased with 20 to 35% of all cancer patients developing brain metastases (Cairncross and Posner, 1983, Posner, 1995). The incidence of brain metastases in breast cancer patients is approximately 15% (Barnholtz-Sloan et al., 2004) with certain breast cancer subtypes, such as $HER2^+$ and triple negative, exhibiting a significantly higher likelihood of recurrence as brain metastases than other subtypes (Kennecke et al., 2010). Despite treatment options, such as whole brain radiation, surgery or stereotactic radiosurgery, and systemic chemotherapy, outcomes remain poor with a median survival time of 4 to 19 months (Ahn et al., 2012). There is, therefore, a need to develop new therapeutic strategies. Given the restrictive nature of the BBB to conventional therapies, new regimes need to include effective drug delivery mechanisms.

Recently, stem cells have been investigated as potential delivery vehicles to treat different CNS diseases. MSCs have been used as cellular delivery vehicles of therapeutic genes to rat glioma (Bexell et al., 2012, Nakamura et al., 2004). Following intratumoural injection, MSCs migrated efficiently within the tumour and did not proliferate. However, systemically administered MSCs exhibited insufficient homing to the brain (Bexell et al., 2012). The role of NSCs as treatment vehicles has been explored for various neurological disorders such as lysosomal storage diseases and stroke (Oh and Choo, 2011). Furthermore, the use of NSCs in animal models of gliomas demonstrated promising results (Aboody et al., 2000). However, intratumoural injection has additional risk for patients and primary autologous NSCs cannot be isolated in the quantities required for the therapy. By contrast, the use of HSCs as treatment vehicles for brain metastases has not previously been investigated. However, recent studies have demonstrated homing of the differentiated progeny of HSCs to diseased brain in animal models as well as in patients (e.g. ALD, multiple sclerosis, Alzheimer's disease, glioma) (Cartier et al., 2009, Ruckh et al., 2012, Malm et al., 2005, Tabatabai et al., 2010). Furthermore, lentivirally transduced human $CD34^+$ HSCs have been used as carriers in a successful phase I/II clinical trial to treat ALD, a severe demyelinating brain disease (Cartier et al., 2009) demonstrating that the progeny of systemically administered HSCs can efficiently home to diseased brain in patients. Primary human HSCs can be isolated in large quantities from cord blood or mobilised peripheral blood. Moreover, the procedures for the clinical use of HSCs are well established and therefore, clinical translation studies using HSCs as delivery vehicles have great promise. Given these data, we hypothesised that the progeny of HSCs could be lentivirally transduced prior to

administration, successfully cross the BBB, and home to metastases in the brain where they could deliver a therapeutic agent.

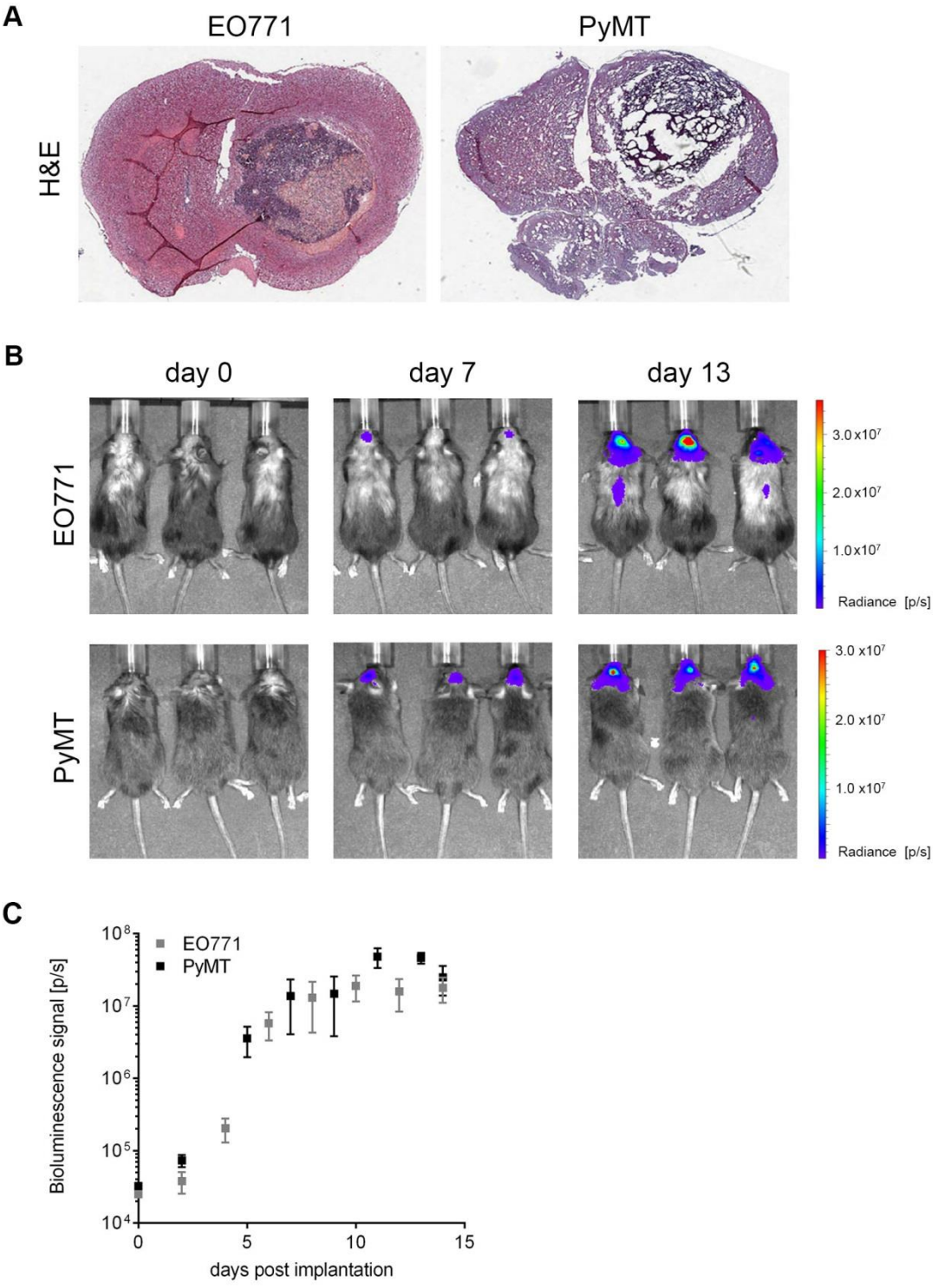
3.2 Aims of the chapter

To test this hypothesis, the aims of this chapter are to:

- Identify and characterise the myeloid cells infiltrating mouse and human breast cancer brain metastases.
- Demonstrate that these myeloid cells originate from bone marrow HSCs.
- Show efficient delivery of a gene of interest to brain metastases by systemically administered lentivirally engineered HSCs.

3.3 Models of breast cancer brain metastases used in the study

Syngeneic brain tumour mouse models were used to study the tumour microenvironment of breast cancer brain metastases and to explore the potential of HSCs as therapeutic vehicles. Firstly, the growth behaviour of two different murine breast cancer cell lines was analysed following intracranial injection. $1x10^5$ EO771 or PyMT cancer cells were implanted into the striatum of C57BL/6J mice. Following implantation, both cell lines grew efficiently and formed large single lesions within two weeks (Figure 3-1A). Prior to the administration, both cell lines were transduced with pFUW-FLuc lentivirus to constitutively express Firefly luciferase (FLuc). The FLuc expression of the cancer cells allowed the non-invasive measurement of brain tumour growth following ip injection of luciferin. Luciferin is a substrate of the enzyme Firefly luciferase and is converted to a product oxyluciferin while emitting light. Bioluminescent images were taken for each mouse (Figure 3-1B) and analysed with LivingImage3.2 software. The brain tumour growth in these mice over time is visualised in Figure 3-1C. Both cell lines exhibited similar growth kinetics during the experiment. Sections of these brain tumour models were also used for immunofluorescence studies of the tumour microenvironment which revealed a strong infiltration of $CD45^+$ cells (Figure 3-1D). We postulate that the $CD45^+$ cells in brain metastases are bone marrow-derived haematopoietic cells, whereas brain-resident microglia have recently been shown to originate from the umbilical vesicle (Ginhoux et al., 2010).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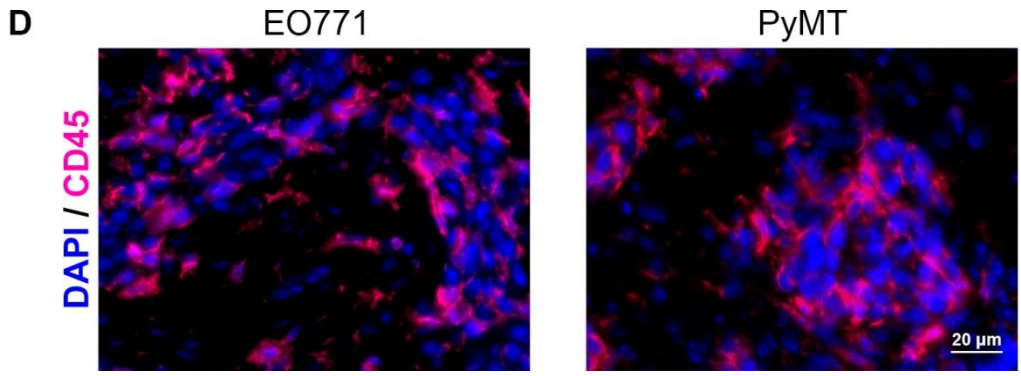


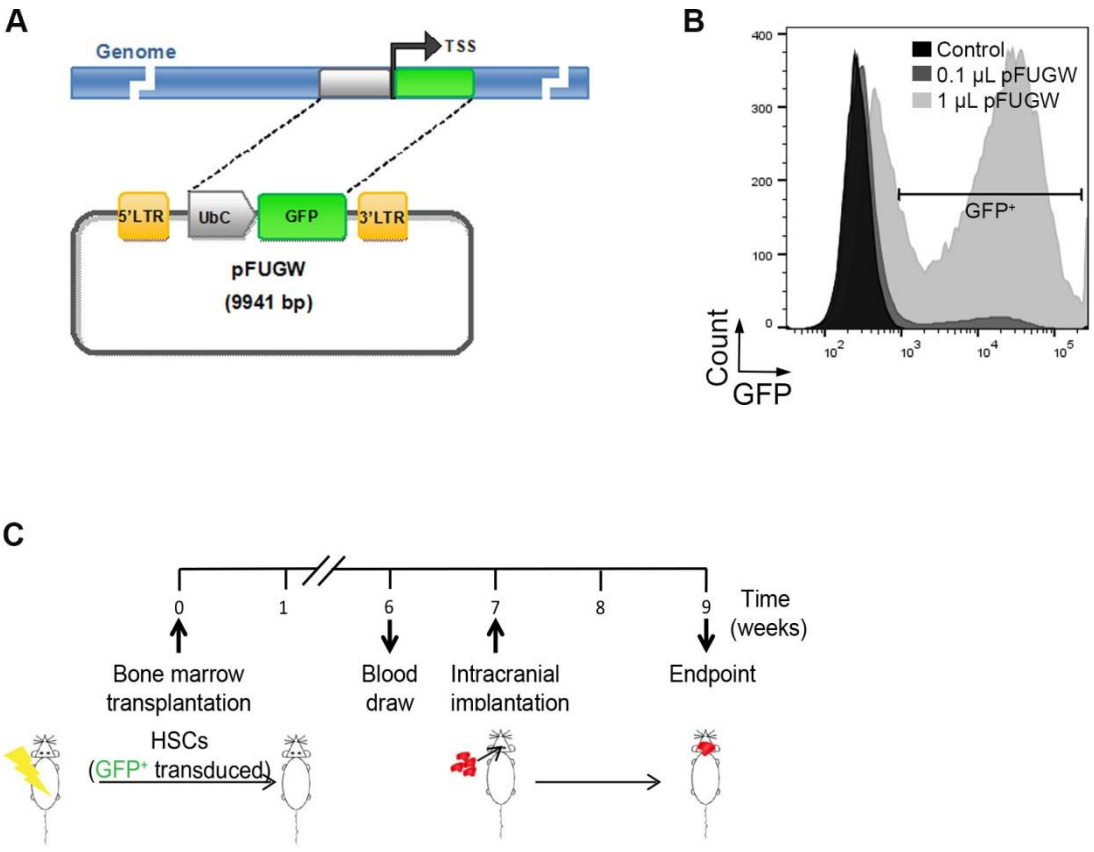
Figure 3-1: Establishment of syngeneic *in vivo* brain metastases models and infiltration of CD45$^+$ cells into murine brain metastases. (A) Following intracranial implantation of EO771 and PyMT breast cancer cells, tumours grew as a single lesion in the brain. **(B and C)** FLuc labelled cancer cells allowed bioluminescent measurement of brain tumour growth following ip injection of luciferin. Bioluminescent images were analysed with LivingImage3.2 software (n=5 mice per group, mean+/-SEM). **(D)** IF analysis shows that EO771 and PyMT cancer lesions in the brain (day 14 post implantation) are strongly infiltrated by CD45$^+$ cells (scale bar= 20 µm, original magnification 40x).

3.4 Bone marrow-derived haematopoietic cells as cell vehicles for targeting of brain metastases

The infiltration of haematopoietic cells into brain metastases led us to investigate whether genetically encoded molecules, introduced into HSCs via lentiviral transduction, could be delivered to brain metastases, following their differentiation to haematopoietic cells. To this end, an experiment using chimeric mice with GFP-tagged bone marrow was performed. The lentiviral vector pFUGW (Lois et al., 2002) was used to stably transduce murine HSCs (Figure 3-2A). To this end, lentiviral particles of pFUGW were first produced and concentrated. The virus titre was determined by flow cytometric analysis of HEK293 cells that have been transduced with different amounts of the virus stock (Figure 3-2B). Once cells were transduced by virus particles, the DNA sequence between the LTRs was integrated into the genome of the cell. The pFUGW lentiviral virus contains the sequence of GFP that is under the control of the UbC promoter (Figure 3-2A). Therefore, the concentration of the virus stock can be measured by the emitted green fluorescence of the transduced cells. Following transduction of $5x10^5$ HEK293 cells with 0.1 µL pFUGW virus stock, flow cytometric analysis showed that 13% of the cells were GFP$^+$ (Figure 3-2B). This implies that the titre of the generated pFUGW virus stock was $6.5x10^5$ virus particles/µL.

In order to generate chimeric mice, the bone marrow in five C57BL/6J mice was ablated by whole body irradiation and then a transplantation of HSCs, lentivirally transduced with

pFUGW at a multiplicity of infection (MOI) of 30 (experimental outline is shown in Figure 3-2C), was performed. Six weeks after the BMT, peripheral blood was analysed by flow cytometry to determine if the bone marrow was successfully reconstituted by transplanted HSCs (Figure 3-2D). 25-45% of all leukocytes in chimeric mice displayed GFP expression, compared with naïve (0.1%) and UbC-GFP mice (92%). Two weeks post intracranial implantation of $1x10^5$ EO771 cancer cells, brain tumours were harvested, singularised and analysed by flow cytometry (n=3) or sectioned and analysed by IF (n=2). The progeny of the genetically engineered HSCs were able to cross the BBB and infiltrate brain lesions, since GFP expressing cells could be detected in brain tumours by IF (Figure 3-2E). Moreover, $CD45^+$ cells could also be detected in brain tumours by flow cytometry (Figure 3-3A). This experiment served as a proof-of-principle to demonstrate that genetically modified HSCs, administered systemically can be utilised as a delivery vehicle in a therapy to treat brain metastases.



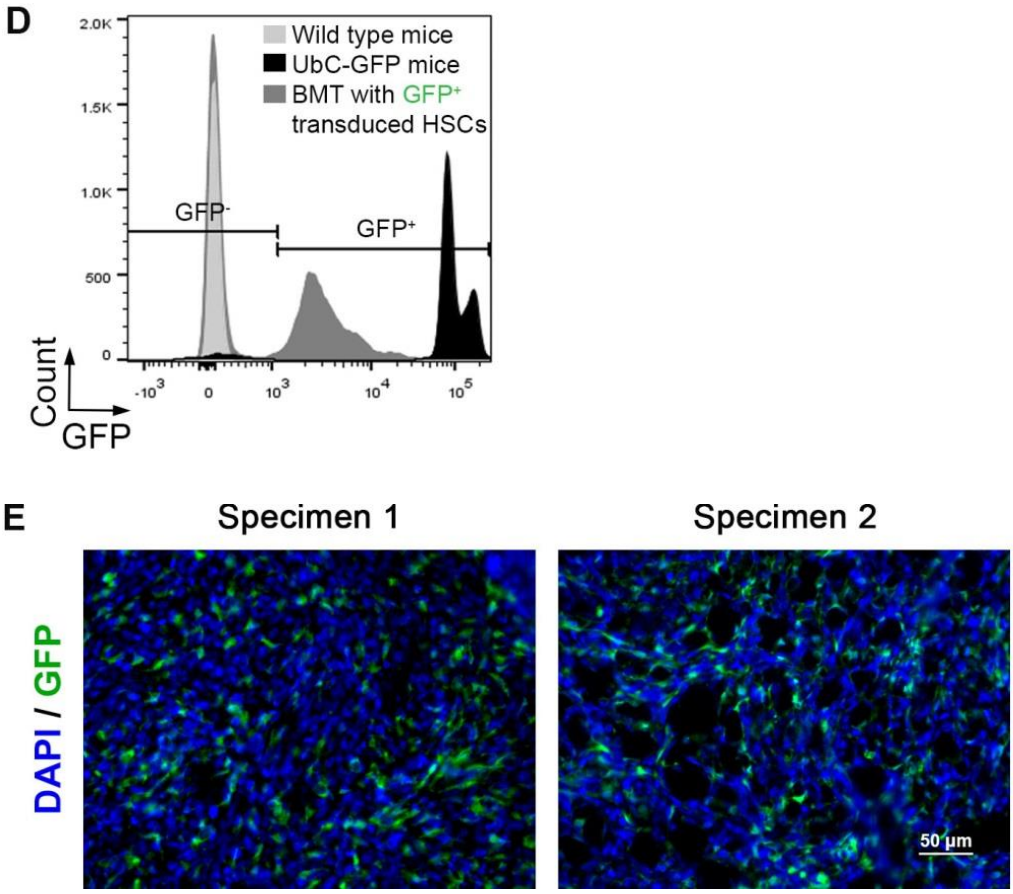


Figure 3-2: The use of genetically modified HSCs and their progeny as cellular delivery vehicles for targeting brain metastases. (A) Illustration of lentiviral expression construct pFUGW used for the *in vivo* experiment (TSS=transcription start site, LTR=long terminal repeats, UbC=Ubiquitin C promoter). **(B)** The virus titre was determined by flow cytometric analysis of HEK293 cells transduced with different amounts of virus stock (1 µL, 0.1 µL and control=non-transduced). **(C)** Experimental outline is shown. Intracranial implantation of EO771 cells was performed 7 weeks after mice underwent BMT with GFP transduced HSCs. **(D)** The blood of the mice was analysed by flow cytometry to confirm bone marrow reconstitution 6 weeks after BMT. **(E)** IF analysis shows that the brain lesions are strongly infiltrated by GFP$^+$ cells (scale bar= 50 µm, n=2 technical replicates, original magnification 20x).

3.5 Characterisation of brain metastases-infiltrating haematopoietic cells in mouse model

To characterise the brain metastases-infiltrating cells, brain tumours and their microenvironment were further investigated in syngeneic brain tumour models. As shown by IF analysis of brain metastases sections from these syngeneic tumour models, the brain lesions showed a strong infiltration of CD45$^+$ cells (Figure 3-1D). CD45$^+$ cells can either represent bone marrow-derived haematopoietic cells or brain-resident microglia. Other markers such as CD11b or F4/80 are also expressed on monocytes/macrophages and microglia in mice. However there is no marker that distinguishes between brain

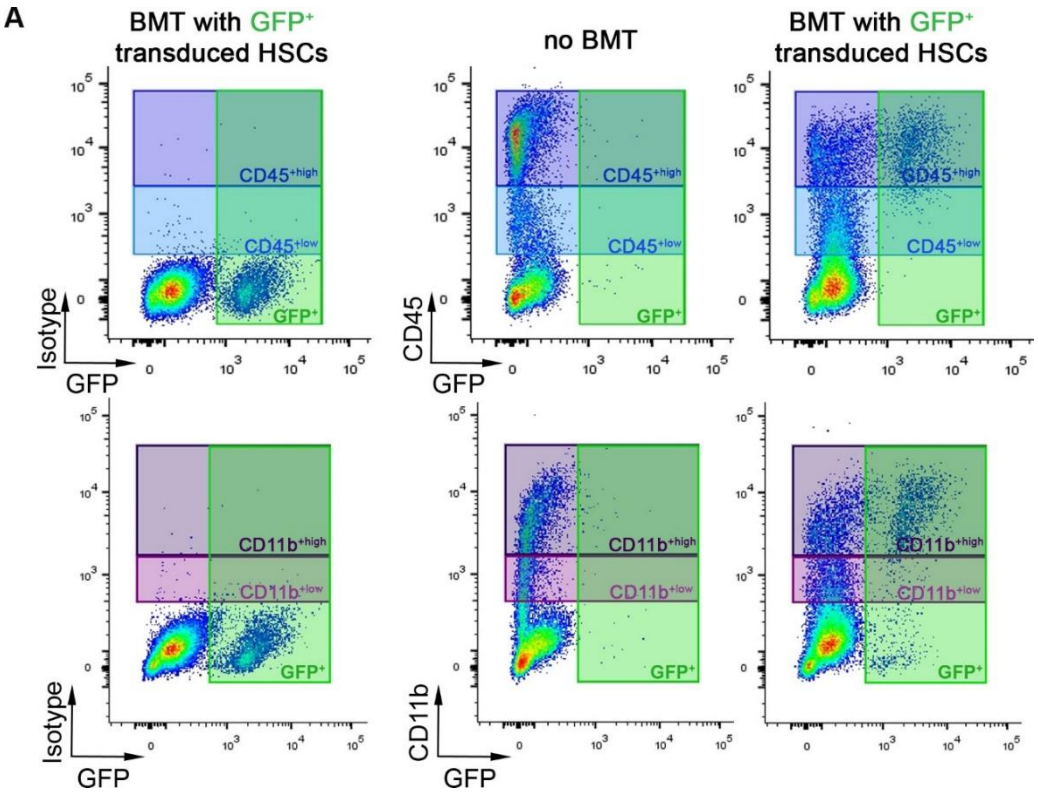
resident microglia and bone marrow-derived myeloid cells via IF, as this method does not discriminate between the different levels of expression of these markers. To determine the proportion of $CD45^+$ and $CD11b^+$ cells in brain metastases that are bone marrow-derived macrophages versus brain-resident microglia, the brain tumours in chimeric mice that received GFP-transduced HSCs (Figure 3-2C and D) were analysed by flow cytometry (n=3).

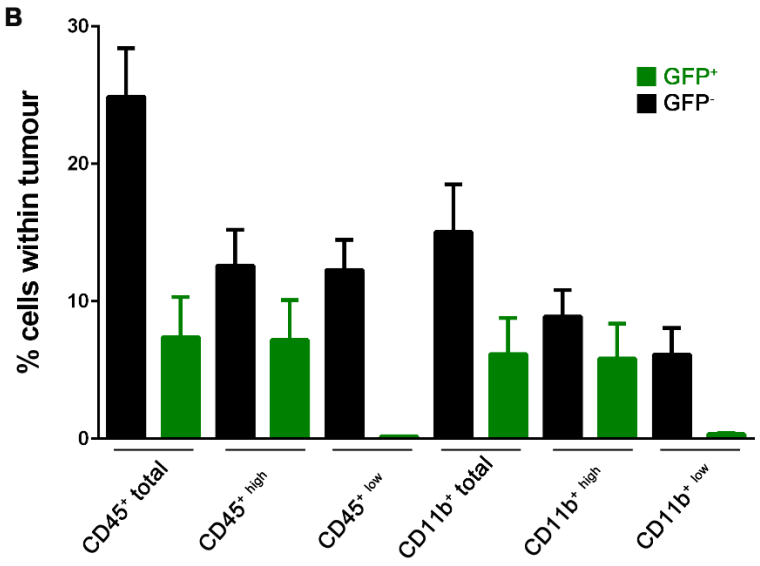
This allowed differentiation between the $CD45^{low}$ and $CD11b^{low}$ brain-resident microglia and the $CD45^{high}$ and $CD11b^{high}$ bone marrow-derived macrophages (Ford et al., 1995, Hickman et al., 2013). Evidently, myeloid cells of the monocyte/macrophage lineage were the most abundant infiltrating cells within brain lesions with 18% $CD11b^{high}$ and 20% $CD45^{high}$ compared with 7% $CD11b^{low}$ and 12% $CD45^{low}$ cells (Figure 3-3A and B). Moreover, GFP was almost exclusively expressed in $CD45^{high}$ and $CD11b^{high}$ cells and absent in $CD45^{low}$ and $CD11b^{low}$ cells, confirming their bone marrow and brain origin, respectively.

Transplanted HSCs give rise to haematopoietic cells of myeloid (monocytes/macrophages, neutrophils, basophils, eosinophils, erythrocytes and megakaryocytes) and lymphoid (T-, B- and NK cells) lineages. To further characterise the bone marrow-derived cells within brain metastases, brain tumour samples were stained for markers of different haematopoietic cell populations and analysed by flow cytometry. As shown in Figure 3-3C and D, the majority of infiltrating cells were $CD11b^+$ myeloid cells, $F4/80^+/CD11b^{high}$ macrophages and $Ly6C^+$ monocytes and activated macrophages. Whereas neutrophils ($Ly6G^+$), granulocytes ($Gr1^+$), T-cells ($CD3e^+$) and B-cells ($B220^+$) represented only minor sub-populations. The ratio between cancer cells and infiltrating $CD11b^+$ myeloid cells was approximately 3:1, demonstrating a strong presence of infiltrating cells. Further quantifications of GFP^+ cells within different haematopoietic cell populations were performed as shown in Figure 3-3C. Approximately, 50% of $CD11b^+$, $F4/80^+$ and $Ly6G^+$ cells expressed GFP, while GFP expression was lower in granulocytes, T-cells and B-cells. This experiment further demonstrated that the genetically engineered HSC progeny homing to brain metastases are capable of delivering genetically expressed molecules to cancer cells within the brain.

All mice underwent a BMT and to demonstrate that this process did not significantly alter the composition of brain tumour-infiltrating cell populations, the brain metastases lesions in naïve mice (i.e. those that did not receive BMT) and mice that underwent a BMT were analysed and compared (Figure 3-3D). Evidence has indicated that irradiation can lead to BBB disruption and therefore increase the permeability of the BBB (reviewed in van

Vulpen et al. (2002)). These microvascular disruptions are sites of inflammation and can therefore attract cells of the immune system. However, no apparent difference in any of the brain tumour infiltrating cell populations between naïve mice and mice that received HSC transplantation could be detected.





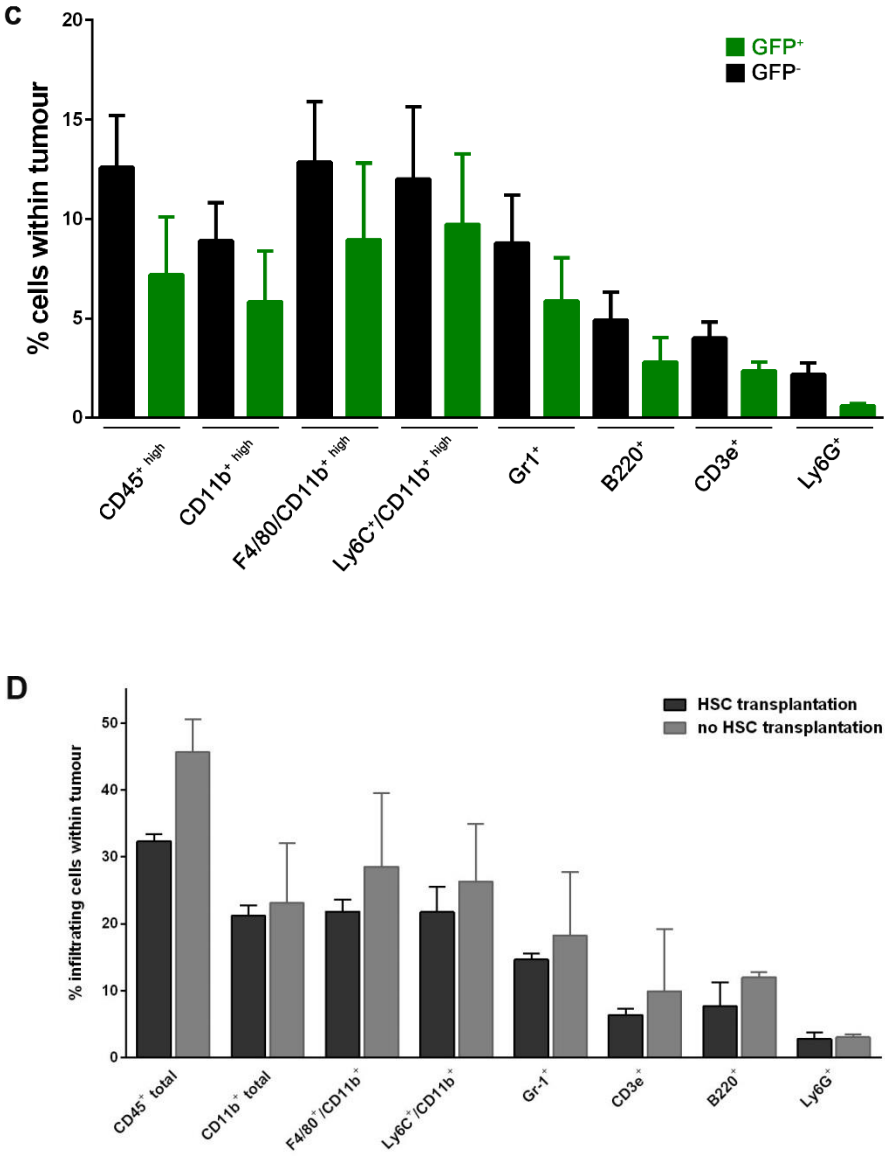


Figure 3-3: Brain metastases infiltrating haematopoietic cells originate from bone marrow and are mainly comprised of macrophages. (A) Brain tumour infiltrative cells were characterised by flow cytometry using different haematopoietic markers (CD45 and CD11b shown only, n=3 technical replicates, BMT= bone marrow transplantation). **(B)** Quantification of (A) shows percentages of brain resident microglia ($CD45^{+low}$ or $CD11b^{+low}$) which do not express GFP and bone marrow-derived myeloid cells ($CD45^{+high}$ or $CD11b^{+high}$). Percentages of GFP-expressing cells are shown in green. **(C)** Quantification of bone marrow-derived haematopoietic cell sub-populations infiltrating brain metastases is shown. Percentages of GFP-expressing cells are depicted in green. **(D)** Further quantification of flow cytometry analysis was performed to demonstrate that the BMT did not lead to an alteration in the composition of brain metastasis-infiltrating haematopoietic cell sub-populations (mice that received BMT: n=3; black bars and wild type mice that did not undergo bone marrow ablation or HSCs transplantation: n=2; grey bars).

3.6 Tumour-associated macrophages in human brain metastases

The major brain tumour infiltrating cell population in the murine *in vivo* models were monocytes/macrophages. To determine to what extent these cells were present in patient-derived brain metastases, four different specimens of human breast cancer brain metastases were examined by IF analysis (Figure 3-4). The tissue sections were stained for CD45, a general haematopoietic cell marker, CD68, a macrophage marker and DAPI, a nuclei marker. Cancer cells were visualised by pan-cytokeratin staining of adjacent sections. This method did not allow a distinction between macrophages and microglia. All human brain metastases specimens were strongly infiltrated by $CD45^+$ cells and elongated spindle-shaped $CD45^+CD68^+$ macrophages. These results correlated with those reported in the *in vivo* murine model experiments.

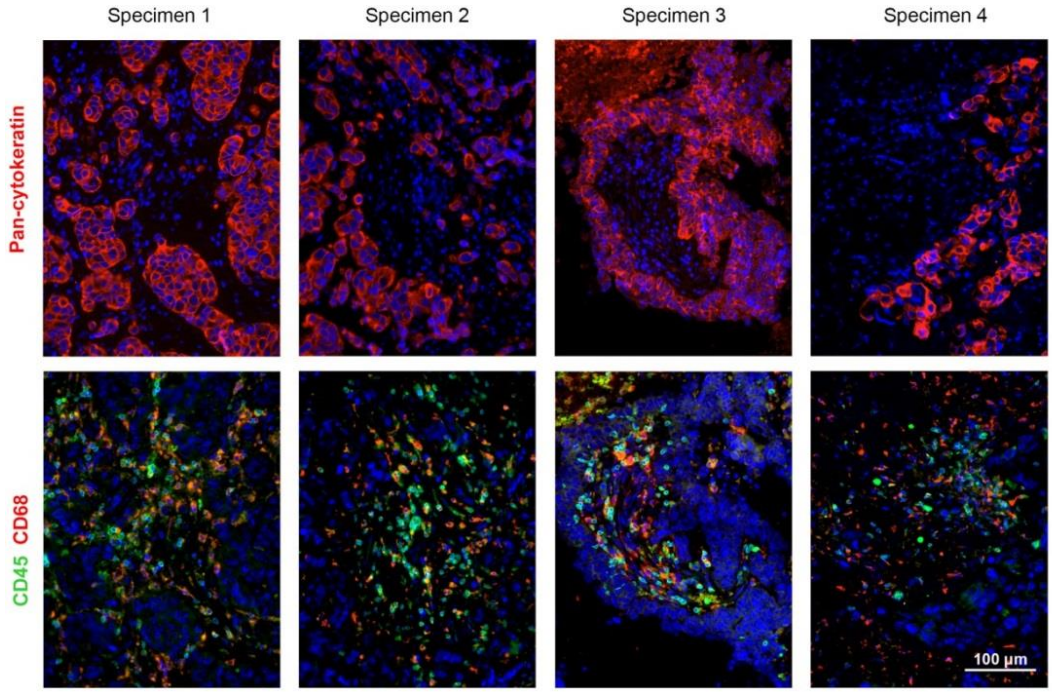


Figure 3-4: Infiltration of TAMs in human breast cancer brain metastases. Adjacent tissue sections of human breast cancer brain metastases samples were stained for pan-cytokeratin (red, upper panel), and co-stained for CD45 (green) and CD68 (red, both bottom panel) and DAPI (blue), (Scale bar = 100 µm, n=4 different patient samples, original magnification 20x).

3.7 Discussion

Treatment options for brain metastases are limited by the poor access of drugs to the brain (Motl et al., 2006, Ohtsuki and Terasaki, 2007). New therapies need to include novel ways of effectively delivering drugs to the central nervous system. The results in this chapter show that myeloid cells originating from HSCs strongly infiltrate brain metastases and could be used as cell vehicles for the delivery of therapeutic agents. The basis of this chapter was the demonstrated strong presence of $CD45^+$ cells in *in vivo* brain metastases models. The infiltration by $CD11b^+$ microglia and macrophages has also been shown previously (Zhang and Olsson, 1995, He et al., 2006, Lorger and Felding-Habermann, 2010). To demonstrate that bone marrow-derived macrophages, and not the umbilical vesicle-derived, brain-resident microglia, were the predominant infiltrating cell population in brain lesions, a phenotypic analysis was performed. This analysis showed that the majority of myeloid cells in brain metastases express high levels of CD45 and CD11b, characteristic of macrophages (Sedgwick et al., 1991, Ford et al., 1995, Davoust et al., 2008). Other markers can also be used to distinguish between microglia and peripheral macrophages, e.g. the expression level of F4/80 (Perry et al., 1985). Macrophages are known to be $F4/80^{hi}$ and microglia to be $F4/80^{lo}$ expressing populations (Gomez Perdiguero et al., 2015). Further markers, that are differentially expressed in macrophages and microglia can be used to define these two populations (reviewed in (Guillemin and Brew, 2004)).

These brain metastases-infiltrating myeloid cells represented a high proportion (approximately 30-50%) of cells within murine brain tumours. Therefore, it can be reasoned that myeloid cells represent promising therapeutic cell vehicles if capable of delivering therapeutic molecules to cancer cells. Additionally, myeloid cells were uniformly distributed throughout the tumour, from the core of the lesion to the tumour border (Figure 3-5). This would result in a uniform distribution of therapeutic agents within the tumour. Taken together, these data confirm that this methodology could be a promising cell-based therapy approach.

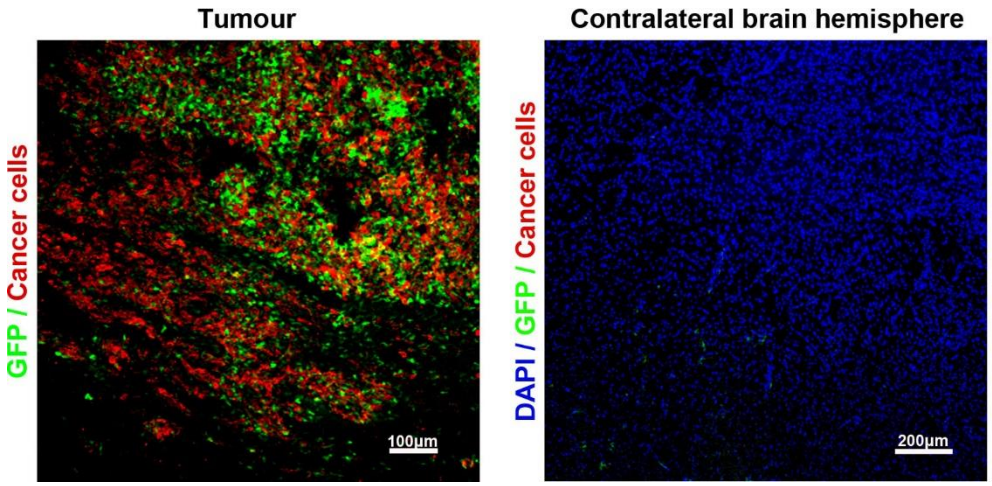


Figure 3-5: Distribution of bm-derived haematopoietic cells within the brain of a brain tumour-bearing mouse. Brain sections of chimeric mice with GFP-tagged bm that received intracranial implantation of red fluorescent labelled EO771 cancer cells were analysed by IF. Nuclei are shown in blue, cancer cells in red and bm-derived cells in green. Experiment and analysis were performed by Mihaela Lorger, original magnification = 10x.

The specificity of the homing of the progeny of HSCs was further examined. Firstly, the myeloid progeny of GFP-tagged HSCs in chimeric mice could not be detected in non-affected, healthy brain tissue after intracranial implantation of cancer cells in mice. This was assessed by IF analysis of brain sections, where no GFP^+ cells could be detected in the contralateral brain hemisphere (Figure 3-5). Secondly, only a minor proportion of the myeloid progeny of HSCs infiltrated wounded brain tissue. More precisely, flow cytometry analysis of brain tissue from sham-operated chimeric animals with GFP-tagged bone marrow, intracranially injected with saline, exhibited only a minor infiltration of GFP^+ cells, as shown in Figure 5-2A. These findings suggest that healthy or wounded parts of the brain would remain unaffected by the cell-based therapy approach.

For the generation of chimeric mice with GFP-tagged bone marrow, a BMT was performed using HSCs that were genetically engineered to express GFP. To this end, HSCs were transduced using the pFUGW lentivirus at an MOI of 30. Eight weeks after the transplantation all animals showed haematopoietic reconstitution and demonstrated an average GFP expression of 44% (range: 31%-54%) in peripheral blood. However, it has been shown in similar experiments that higher reconstitution rates can be achieved. Tabatabai and colleagues could detect 80-92% GFP^+ cells in peripheral blood of mice that underwent a bm engraftment using haematopoietic progenitor cells (HPC) that were lenti-GFP transduced at an MOI of 100 (Tabatabai et al., 2010). It has also been shown that RBCs of mice expressed 96% human $\beta^{A\text{-}T87Q}$-globin protein on average 10 months after BMT with HSCs, that have been transduced with human $\beta^{A\text{-}T87Q}$-globin lentivirus (Pawliuk et al., 2001). Therefore, it is possible that in order to achieve higher transduction

efficiency the transduction protocol could be modified, e.g. increasing the MOI, repetition of the transduction process or prolonging the transduction duration (Millington et al., 2009). Further, the transduction efficiency can also be increased by performing a spinoculation (O'Doherty et al., 2000). Moreover, the presence of positively-charged polycations, such as polybrene, can reduce the electrostatic repulsion forces between a negatively-charged cell and an approaching enveloped lentiviral particle resulting in an increase in transduction efficiency (Toyoshima and Vogt, 1969). Other polycations, such as protamine sulfate and diethylaminoethylcellulose (DEAE)-dextran demonstrated superior results to polybrene in enhancing lentiviral transduction of most tested cell lines and primary cell cultures (Cornetta and Anderson, 1989, Denning et al., 2013).

A major drawback of this HSC-based therapy approach is the low intracranial specificity of the delivery due to the accumulation of HSC progeny in organs such as the spleen, lungs, liver, blood and bone marrow. This issue is addressed in chapter 5 of this study.

The bone marrow-derived myeloid cells within the brain metastases have been further characterised to determine which cell population they are mainly composed of. Flow cytometric analysis of murine brain metastases revealed F4/80^{+}/CD11b^{high} macrophages and Ly6C^{+} monocytes to be the major tumour-infiltrating myeloid cell population. In agreement with this, macrophages/microglia were also found to be the main infiltrating population in human breast cancer brain metastases. Macrophage infiltration of clinical and experimental brain metastases was reported more than two decades ago (Morantz et al., 1979, Schackert et al., 1988). However, to date it is largely unknown how macrophages are recruited by brain metastases (Strik et al., 2004). TAMs can either be in an M1 (tumour-suppressive) or M2-like state (tumour-supportive). Previous work has shown that TAMs mainly acquire M2-like properties in most cancers with potent immunosuppressive functions (Mantovani et al., 2002). A whole genome analysis of murine brain infiltrating myeloid cells was performed, which will be explained in more detail in chapter 5 of this study. This experiment revealed that murine TAMs overexpress a variety of M2 markers, e.g. Arg1, MMPs and chemokines like CXCL16, whereas a downregulation of M1 markers, e.g. IL-12, IL-23, IFN-ɣR, CD80 and CD86, could also be detected (according to Biswas and Mantovani (2010)). However, conclusions on the polarisation state of TAMs can only be made by looking at murine and human brain metastases. Therefore, macrophage classification analysis on human breast cancer brain metastases TAMs needs to be performed. However, current data indicates that the over-expression of several M2 markers and down-regulation of M1-markers in murine brain tumour TAMs and, thus, suggests an M2-like macrophage phenotype. Given the anti-inflammatory, pro-angiogenic and tumour-promoting properties of M2-like TAMs and

the large quantities of brain tumour infiltrative macrophages, it is possible that re-polarisation or therapeutic deletion of these cells could be a possible therapeutic option that could be combined with the cell-based therapy. Methods to re-polarise TAMs from an M2 to an M1-like state have been reported in various pre-clinical studies. These include the use of a legumain-based DNA vaccine which induced a robust $CD8^+$ T cell response against TAMs and led to reduced tumour angiogenesis, tumour growth and metastasis formation in breast cancer models (Luo et al., 2006). Other strategies to re-polarise TAMs include the use of histidine-rich glycoprotein (Rolny et al., 2011), interferon-gamma (Duluc et al., 2009), the blockade of nuclear factor-kB signalling (Hagemann et al., 2008) or their exposure to anti-IL-10R antibodies combined with the TLR9 ligand CpG (Guiducci et al., 2005). However, further investigation into the effects of re-polarisation therapies on other leukocyte populations and the potential off-target effects still need to be addressed.

The application of monocytes/macrophages as a cellular delivery vehicle has already been demonstrated in the context of other brain diseases. Afergan et al. used monocytes that phagocytosed nanoliposomes and thereby delivered serotonin across the BBB (Afergan et al., 2008), whereas Dou and colleagues demonstrated the application of macrophages as carriers for anti-retroviral drugs (Dou et al., 2009) to treat a HIV-associated neurocognitive disorder. Recently, nanoparticles were successfully delivered to breast cancer brain metastases by monocytes/macrophages following systemic administration. However, the injected monocytes/macrophages showed a similar distribution to endogenous macrophages and resided not only in brain metastases but also in lungs and liver (Choi et al., 2012). Therefore, to avoid toxicity in healthy tissue, a therapy that specifically targets brain tumour cells or is specifically activated within the tumour microenvironment would need to be delivered by these nanoparticles.

Overall, the results of this chapter provide a basis for the targeting of brain metastases with HSC therapy and demonstrate its potential for translation into the clinic. Firstly, murine brain metastases exhibited significant levels of tumour infiltration by myeloid cells of bone marrow origin. Secondly, it was demonstrated that the myeloid progeny of HSC, genetically engineered to express GFP, can efficiently deliver GFP to brain lesions following engraftment. Finally, patient-derived brain metastases specimens displayed a strong infiltration of TAMs, correlating with findings in murine models of brain metastases. This data demonstrates the translational potential of this therapeutic approach. The next chapters of this study will focus on the development of an enzyme prodrug approach for HSC-based cell therapy and a strategy to improve the specificity of this therapy.

4. Development of an enzyme prodrug approach as a therapeutic option for HSC-based cell therapy to treat brain metastases

4.1 Introduction

The tumour-homing capability of the progeny of systemically administered HSCs demonstrated their suitability to act as delivery vehicles of therapeutic molecules to target brain metastases. To limit the toxic effect of the delivered therapeutic molecule at the tumour site, the therapy strategy needs to be chosen carefully. An enzyme prodrug therapy presents a good option. It is a two-step approach. Firstly, a drug-activating enzyme is expressed either by cancer cells or cells within the tumour microenvironment. Secondly, the non-toxic prodrug is systemically administered and is converted to the active drug within the tumour. To be successful, both the enzyme and the prodrug must meet certain requirements. The prodrug should be a good substrate for the expressed enzyme in the tumour tissue. In addition, it should not be activated by endogenous enzyme in non-tumour tissues. To exert a bystander killing effect, the activated drug is expected to be highly diffusible or actively taken up by adjacent cells (Niculescu-Duvaz et al., 1998). Furthermore, to induce the bystander effect, the half-life of the activated drug needs to be of an appropriate length but short enough to prevent the drug from leaking into the systemic circulation (Niculescu-Duvaz et al., 1998). The prodrug converting enzyme should either be of non-human origin or a human enzyme only expressed at low concentrations in normal tissue (Rainov et al., 1998, Xu and McLeod, 2001). In addition the enzyme should have a high catalytic activity and be expressed extensively within the tumour (Niculescu-Duvaz et al., 1998).

One of the most widely used enzyme prodrug approaches is cytosine deaminase (CD), derived from yeast or bacteria combined with 5-fluorocytosine (5-FC). Cytosine deaminase is not expressed in mammals but can convert the non-toxic prodrug 5-FC to the toxic compound 5-fluorouracil (5-FU) resulting in the inhibition of cancer cell growth. The use of 5-FC as an enzyme prodrug therapy for brain tumours has already been evaluated by others (Mullen et al., 1992, Dong et al., 1996, Ge et al., 1997, Aghi et al., 1998, Wang et al., 1998, Aboody et al., 2006). 5-FC was found to have high bioavailability and could penetrate the blood-brain barrier and enter the CNS (Bourke et al., 1973, Block and Bennett, 1974).

There are currently two major delivery methods for an enzyme prodrug strategy. It can either be delivered by genes that encode prodrug-activating enzymes or directly by delivering active enzymes into tumour tissues (antibody-directed enzyme prodrug therapy (ADEPT)). To deliver genes encoding the prodrug-activating enzymes a gene-directed enzyme prodrug therapy (GDEPT) or a virus-directed enzyme prodrug therapy (VDEPT) can be used. VDEPT is the most suitable method for the HSC-based cell

therapy developed in this study, as GDEPT and ADEPT methods are limited by the properties of the BBB. VDEPT uses viral vectors to deliver a gene that encodes the prodrug converting enzyme. Several viruses have been used for VDEPT, including retrovirus, adenovirus, HSV, EBV and lentivirus (reviewed in Xu and McLeod (2001)). However, lentiviruses are a subclass of retrovirus that can transduce dividing and non-dividing cells, whereas retrovirus and adenovirus can only transduce dividing cells. Following transduction with lentiviral particles, lentiviral vectors are stably integrated in the host cell genome at a random position providing long term transcription of the gene of interest. For safety reasons lentiviral vectors are replication deficient. To produce lentiviral particles, a cell line is transfected with several plasmids, containing genetic material encoding different virion proteins and the gene of interest.

The use of VDEPT engineered adult stem cells as delivery vehicles for enzyme prodrug therapies to target cancer has been shown by others. Systemic administration of MSC expressing a prodrug-converting gene in combination with an appropriate prodrug was demonstrated in glioblastoma, melanoma, prostate, colon and hepatocellular carcinoma model (Altaner et al., 2014, Kucerova et al., 2008, Cavarretta et al., 2010, Niess et al., 2011). Zhao et al. demonstrated an effective prodrug enzyme therapy that was delivered by NSC targeting extracranial metastatic breast cancer effectively (Zhao et al., 2012). Other studies have shown NSCs can deliver therapeutic gene products to tumour cells across the BBB, including glioma, melanoma brain metastases, and disseminated neuroblastoma (Aboody et al., 2000, Benedetti et al., 2000, Aboody et al., 2006, Danks et al., 2007).

4.2 Aims of the chapter

From the reviewed literature an enzyme prodrug approach delivered via VDEPT to engineer HSCs is an appropriate choice for the cell therapy targeting breast cancer brain metastases. This chapter shows how:

- The CD/5-FC therapy was developed and the functionality was assessed *in vitro* and *in vivo*.
- An inducible CD/5-FC therapy was developed and the functionality was assessed *in vitro* and *in vivo*.

4.3 The non-inducible enzyme prodrug approach

4.3.1 Generation of lentiviral constructs for the expression of CD

To establish a cell therapy approach based on an enzyme prodrug strategy, lentiviral expression constructs of the prodrug converting enzyme cytosine deaminase were generated (Figure 4-1A). A two-step PCR was used to add short sequences to the bacterial CD gene sequence using plasmid pORFCodA (InvivoGen) as a template. The forward primer sCD fwd (Table 2-3) introduced a signal peptide sequence (tissue plasminogen activator (tPA)-signal) at the 5´-end of the enzyme sequence. To detect the expression of the enzyme *in vitro* as well as *in vivo*, a HA-tag was attached to the C-terminal end of the enzyme using the reverse primer sCDHA rev (Table 2-3). Another non-tagged sCD PCR product was generated using a reverse primer without the tag sequence. Both PCR products (sCD: 1388 bp and sCDHA: 1451 bp) are shown in Figure 4-1B as detected by agarose gel electrophoresis. The lentiviral vector pFUW was cut using the restriction enzyme HpaI, which led to the linearisation of the plasmid as shown in Figure 4-1B. To insert the PCR products into the linearised vector pFUW, subsequent cloning steps were performed as described in chapter 2.2. Successful incorporation of sCD constructs into pFUW vector was confirmed by restriction digest using the restriction enzymes BamHI and NdeI. Subsequently, DNA sequences of positive clones were verified by sequencing. Sequencing of the HA-tagged clone revealed a presence of seven HA-tags at the C-terminal end of the generated construct, possibly as a result of the PCR annealing temperature being too low and thereby leading to unspecific binding of reverse primer and an unwanted prolongation.

4.3.2 Analysis of sCD expression and secretion

Following generation of lentiviral sCD expression vectors, a lentiviral virus stock was generated for pFUWsCD and pFUWsCDHA constructs and used to transduce HEK293 cells. To determine the expression and secretion of the 5-FC converting enzyme CD in the transduced cells, Western blot analysis of the cell lysates and non-concentrated and concentrated cell culture supernatants was performed (Figure 4-1C). The same amount of protein (20 µg/well) for cell lysates and the same volume of the supernatant samples (30 µL) were loaded into each well of the 10% SDS polyacrylamide gel. Detection was performed with α-HA antibody (1:1000). Western blot analysis showed an expression of the HA-tagged sCD enzyme (expected size: 47.59 kDa; UniProtKB: P25524) in

transduced HEK293 cells. However, secretion of sCD into the medium could not be detected.

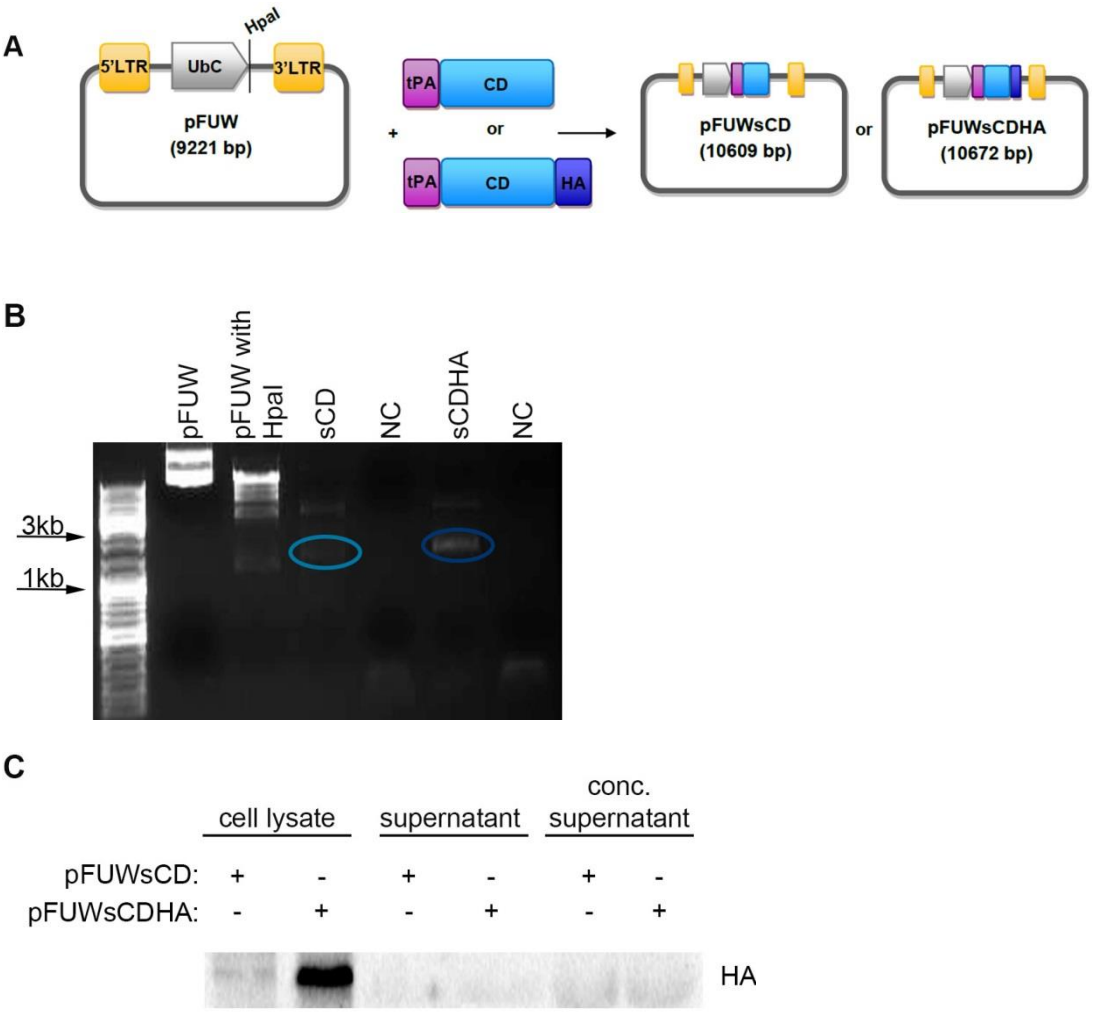


Figure 4-1: Generation of lentiviral expression constructs for sCD and assessment of *in vitro* functionality. (A) Illustration of cloning strategy for the generation of pFUWsCD and pFUWsCDHA (LTR=long terminal repeats, UbC=Ubiquitin C promoter, CD= Cytosine deaminase, tPA= tissue plasminogen activator signal sequence) **(B)** sCD PCR products (sCD: 1388 bp, sCDHA: 1451 bp) generated using pORFCodA vector as a template by two step PCR (lanes 4,6), lentiviral expression vector pFUW uncut and cut with restriction enzyme HpaI (lanes 2,3), and negative controls (NC) for PCR (lanes 5,7) were analysed by agarose gel electrophoresis. GeneRulerTM DNA Ladder Mix (Thermo Scientific) was used as a molecular weight marker (lane 1) **(C)** sCD expression and secretion analysis in pFUWsCD and pFUWsCDHA-transduced HEK293 cells by Western blot using an α-HA antibody.

4.3.3 Assessment of *in vitro* functionality of the sCD expression constructs

The pFUWsCD and pFUWsCDHA transduced HEK293 cells were used for further *in vitro* analysis. A co-culture assay was performed to test whether sCD in carrier cells could kill cancer cells. HEK293 cells were used as carrier cells in this *in vitro* assay. To this end, sCD and sCDHA transduced HEK293 cells were co-cultured with murine breast cancer cells (EO771 or PyMT) in cancer cell-specific medium for four days. The ratio of cancer cells to HEK293 cells was 1:1. The cancer cells constitutively expressed FLuc, which enabled the indirect measurement of cell viability via bioluminescence imaging following addition of luciferin. The medium was changed daily, followed by addition of 0.1 mM 5-FC and 7.5 µg luciferin to the cells 10 min prior to imaging. The results of this experiment are displayed in Figure 4-2A and C. The cancer cells co-cultured with HEK293 cells grew exponentially over the course of the experiment. Furthermore, neither luciferin nor 5-FC were toxic to the cells. A significant difference in viability between EO771 cancer cells that had been co-cultured with sCD-transduced HEK293 cells and then treated with the prodrug 5-FC versus non-treated cancer cells was observed at day three and four of the experiment (Figure 4-2A). The data demonstrated that the non-toxic agent 5-FC was converted into the toxic 5-FU by sCD, expressed by pFUWsCD-transduced HEK293 cells, inducing the death of cancer cells. This effect could also be seen with PyMT cells on day four (Figure 4-2C). This experiment proves the *in vitro* functionality of the lentiviral expression construct pFUWsCD.

To confirm that the results of the co-culture assay were not solely due to bystander effect but were reliant on enzyme secretion by the producing cell, a secretion assay was performed. While Western blot analysis of the supernatant collected from transduced HEK293 cells indicated that the pFUWsCDHA-transduced cells did not secrete the enzyme (Figure 4-1C), the secretion of non-tagged sCD by Western blot could not be detected due to the lack of appropriate antibody. Therefore, this assay was used to indirectly test the secretion of the non-tagged sCD into medium. For this assay Fluc expressing cancer cells were cultured with conditioned medium (CM) collected from sCD and sCDHA transduced HEK293 cells (Figure 4-2B and D). The medium was changed daily, followed by addition of 0.1 mM 5-FC and 7.5 µg luciferin to the cells 10 min before bioluminescence imaging. The results of the imaging demonstrated that sCD transduced HEK293 cells secreted the enzyme into the medium which then converted 5-FC to the toxic compound 5-FU. Cell viability was significantly lower in both cancer cell lines cultured with CM collected from sCD-transduced HEK293 cells following addition of 5-FC on day three and four of the assay. By contrast, sCDHA-transduced HEK293 cells

did express the enzyme (Figure 4-1C), but it appeared to be non-functional since no reduction in cancer cell viability could be detected (Figure 4-2A and C). The secretion assay confirmed the results of the previous Western blot, suggesting that the HA-tagged version of CD was expressed, but not secreted by the sCDHA-transduced HEK293 cells. This might be due to the length of the HA-tag (7x), which could hinder secretion and enzymatic activity of sCDHA via conformational changes of the protein caused by the length of tag at the c-terminal end of the enzyme.

These experiments demonstrate the *in vitro* functionality of the generated lentiviral expression construct pFUWsCD. HEK293 cells produced and secreted the functional enzyme CD following lentiviral transduction with pFUWsCD. Therefore, this expression construct was subsequently applied as part of the cell therapy approach to treat *in vivo* brain metastases models.

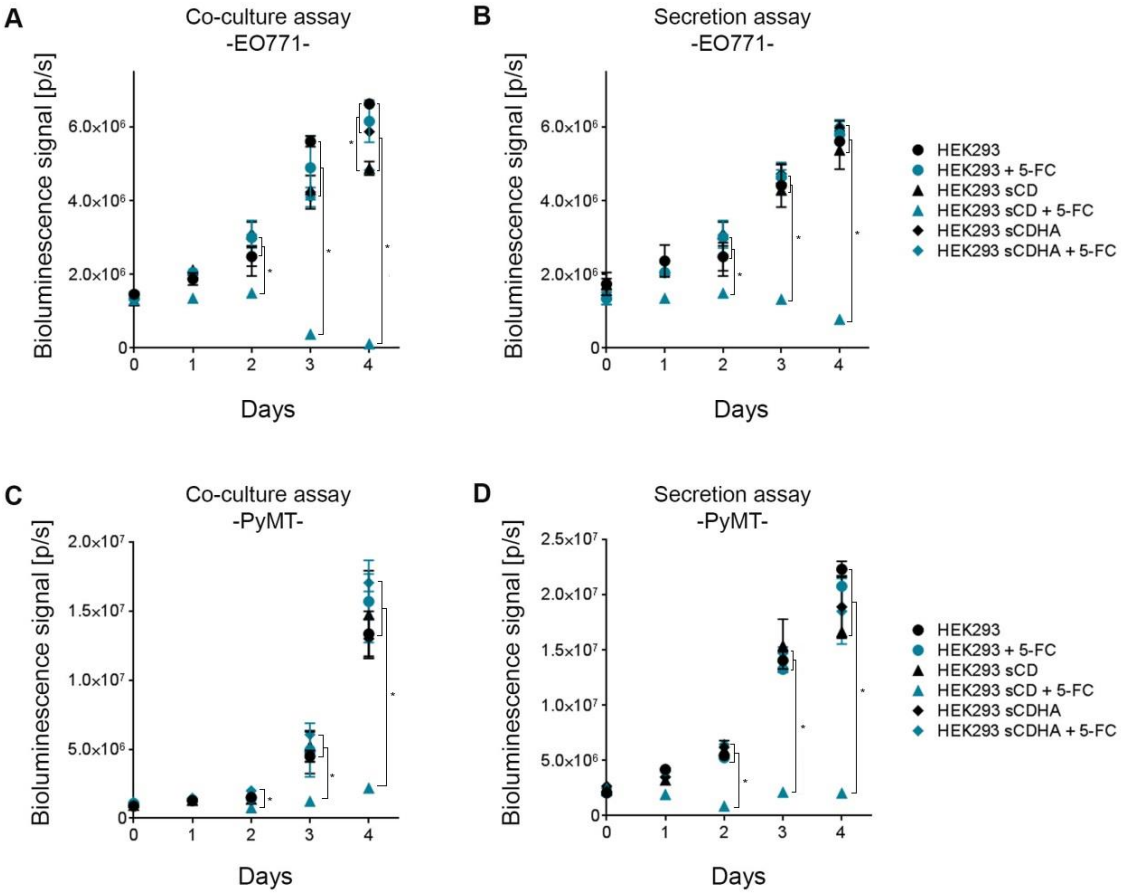


Figure 4-2: Assessment of *in vitro* functionality of lentiviral expression constructs for CD. (A and C) sCD and sCDHA transduced HEK293 cells were co-cultured with FLuc expressing cancer cells (EO771 or PyMT) for 4 days. Bioluminescence signal of cancer cells was measured following addition of luciferin to the medium daily. Only sCD transduced HEK293 cells expressed functional CD, as following daily dosage of 0.1 mM 5-FC a reduced bioluminescence intensity was detected (n=3 technical replicates). **(B and D)** FLuc expressing cancer cells (EO771 and PyMT) were cultured with CM from sCD or sCDHA transduced HEK293 cells for four days. Bioluminescence activity of cancer cells was measured following daily addition of luciferin. The sCD transduced HEK293 cells expressed and secreted CD into culture medium which led to a reduced bioluminescence signal of CM-cultured cancer cells (n=3 technical replicates, mean+/- SEM). Two repeats of the experiment were performed, analysis of one is shown here. Statistical significance was determined with two-way ANOVA method, with alpha=5%.

4.3.4 Testing the functionality of non-inducible enzyme prodrug approach *in vivo*

To test the *in vivo* functionality of the generated lentiviral expression construct pFUWsCD, the following experiment was carried out (Figure 4-3A). A bone marrow transplantation was performed in six female C57BL6/J mice. To this end, $ScaI^{+}$ HSCs were isolated via MACS from the bone marrow of transgenic UbC-GFP mice. The

isolated cells were transduced overnight with concentrated pFUWsCD virus at an MOI of 30. The viral titre of the pFUWsCD virus stock was determined using the Reverse Transcriptase assay (Roche). 24 hours after whole body irradiation was performed, the six mice received an iv injection of $1x10^6$ sCD-transduced GFP^+ HSCs. Six weeks after the BMT, blood was drawn from the tail vein of the mice and analysed by flow cytometry (Figure 4-3B). This was to determine if the bone marrow had been successfully reconstituted by the transplanted HSCs. Approximately 75-91% of all leukocytes in chimeric mice expressed GFP, compared with wild type (0.5%) and UbC-GFP mice (92%).

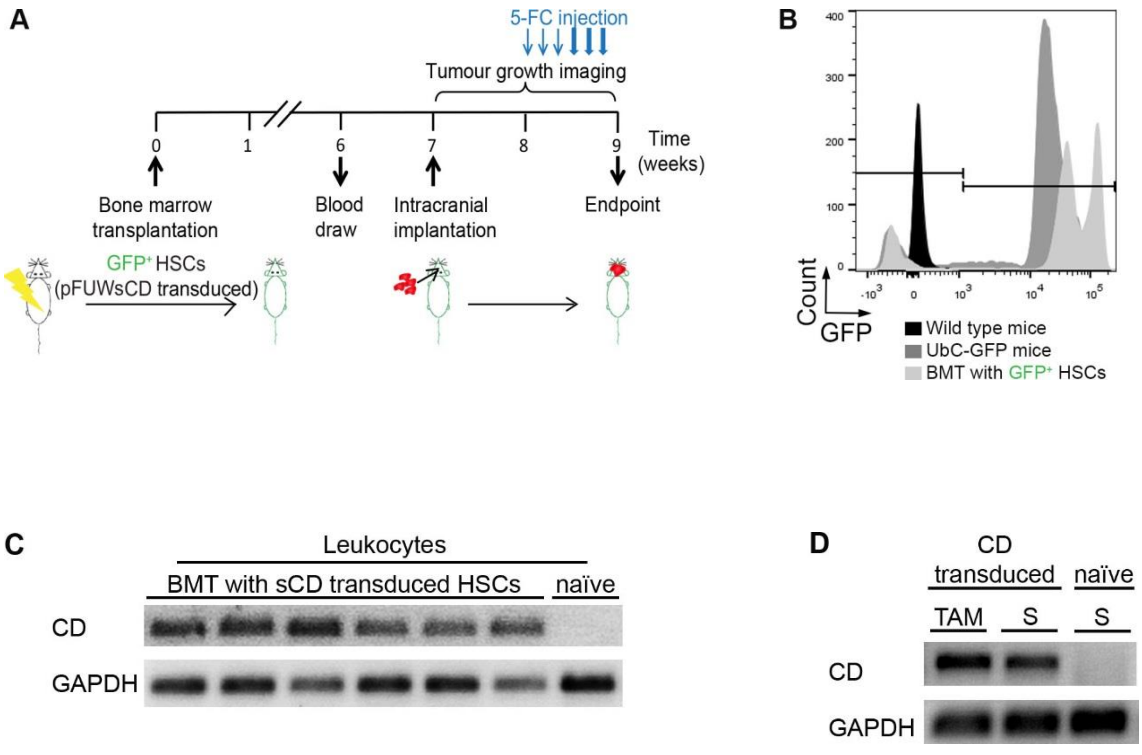
To verify that leukocytes were also expressing sCD, RNA was extracted and a RT-PCR was performed (Figure 4-3C). Results showed that leukocytes of all six mice that had bm engraftment with sCD-transduced HSC expressed the enzyme. Based on these results, the experiment was continued with the implantation of $1x10^5$ EO771/FLuc cancer cells into the mice brains. The FLuc expression in cancer cells enabled the non-invasive measurement of brain tumour growth over time. The brain tumour growth of these six mice is shown in Figure 4-3D. Six days post implantation the mice were grouped, based on similar distribution of bioluminescence tumour signal across both groups. One group received daily ip injections of 900 µL PBS, whereas the other mice received daily injections of 500 mg/kg 5-FC. There was no therapeutic effect on brain tumour growth after three days of treatment, so the dosage was doubled from day nine post implantation. After a further five days of treatment, there was still no effect on the tumour signals and the experiment was terminated.

To investigate why the enzyme prodrug therapy was not successful, the expression of CD in TAMs and tissue resident macrophages was examined. $CD11b^+$ macrophages were isolated via MACS from the brain tumours and the spleens of mice that had received the treatment, and macrophages from naïve mice were used as a control. The RNA of the macrophages was analysed for sCD expression by RT-PCR. As shown in Figure 4-3E, macrophages from tumour and spleen, expressed sCD, although no therapeutic effect could be seen.

Because RT-PCR only allows RNA expression analysis in a qualitative way, a qPCR was performed to have a closer look at the expression levels of sCD in leukocytes. To this end a TaqMan qPCR using sCD-specific probe was performed and the expression of the house keeping gene GAPDH was used as a normalisation control for each sample. HSCs that had been freshly transduced with lentiviral sCD construct were used as a control for this experiment. Figure 4-3F shows that leukocytes of mice that underwent a BMT with pFUWsCD-transduced HSCs displayed extremely low expression levels of

sCD compared with freshly transduced HSCs. This may explain why sCD expression could be shown in TAMs and in leukocytes by RT-PCR, although no therapeutic effect on brain tumour growth was detectable. It is possible that the expression level of the enzyme in TAMs was too low to convert a sufficient amount of 5-FC into 5-FU resulting in no detectable cancer cell death.

As sCD is secreted, the progeny of sCD-transduced HSCs expressing high amounts of the bacterial enzyme could have led to an immune reaction against sCD-expressing cells. If this hypothesis were true, only leukocytes expressing sCD at a low level would have been able to escape the immune response and remain in the mice. Therefore, it was reasoned that in order to circumvent the potential immune reaction against sCD-expressing leukocytes, an inducible expression system could be used. This is addressed in the following chapter.



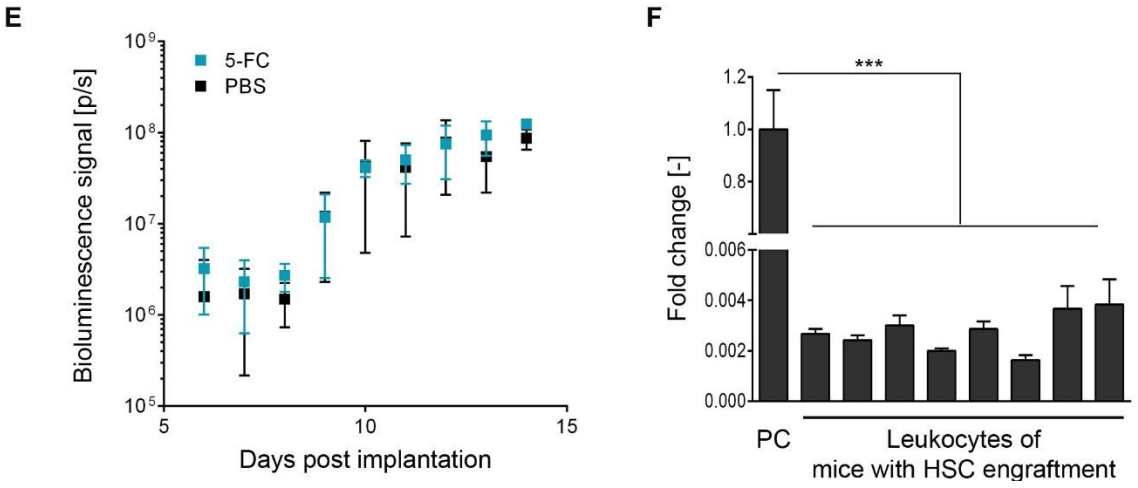


Figure 4-3: Testing functionality of non-inducible enzyme prodrug approach *in vivo*. (A) Schematic overview of the experiment. Mice received GFP$^+$ HSCs from transgenic UbC-GFP. These HSCs were transduced with pFUWsCD lentivirus. Six weeks post-bm transplantation, blood was analysed to confirm reconstitution. Seven weeks after BMT, intracranial implantation of EO771/FLuc cancer cells was performed and the experiment was terminated two weeks later. **(B)** Reconstitution of UbC-GFP HSCs in the blood of C57BL6/J mice was analysed by flow cytometry. **(C)** RNA isolated from leukocytes of naïve mice and mice that received BMT was analysed by RT-PCR to check for CD expression. **(D)** RT-PCR using RNA obtained from CD11b$^+$ cells isolated from tumour tissue and spleen was performed to test CD expression (S=spleen). **(E)** Brain tumour growth was analysed by bioluminescence imaging over time (n=3 technical replicates, mean+/-SEM). **(F)** qPCR using RNA obtained from leukocytes of mice that underwent HSC engraftment was performed to check for CD expression (eight biological leukocyte samples and two freshly sCD-transduced HSCs samples=PC, mean+SEM). Statistical significance determined with paired two-tailed t-test, $p<0.0001$.

4.4 Development of an inducible enzyme prodrug approach

4.4.1 Generation of lentiviral constructs for inducible sCD expression

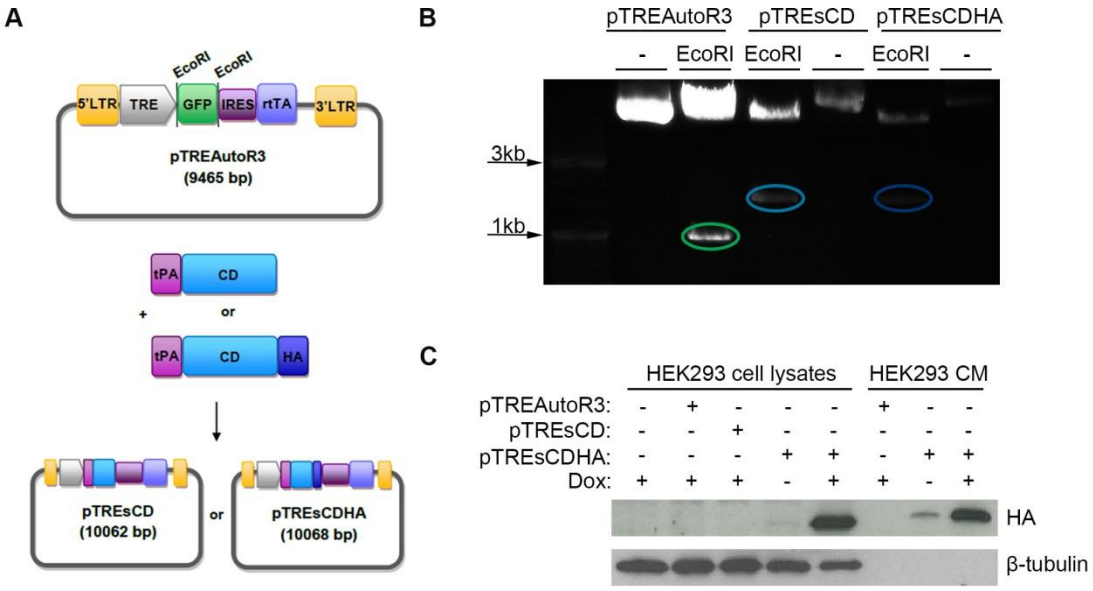
To circumvent a potential immune response against CD-expressing leukocytes, inducible lentiviral expression constructs for sCD were generated using the vector pTREAutoR3 (Markusic et al., 2005) as the backbone. This doxycycline (Dox) inducible vector has been used in a stem cell gene therapy for breast cancer (Li et al., 2009). It contains a bicistronic cassette composed of GFP and the tetracycline-responsive transactivator (rtTA) under the control of a minimal CMV promoter linked to tetracycline-responsive element (TRE) (Figure 4-4A). Once Dox is added to this system, it binds together with rtTA to the TRE and initiates transcription of the cassette. To bring sCD expression under the control of the Dox-inducible promoter, the GFP sequence in pTREAutoR3 was replaced by the sCD sequence isolated from pFUWsCD (Figure 4-4A). Because the HA-tagged version of sCD in pFUWsCDHA was expressed but not functional, a new construct for HA-tagged sCD was generated. To this end, a two-step PCR was performed with primers tPA_NdeI fwd and sCDHA 2 rev (Table 2-3) using pFUWsCD as the PCR template. To insert sCDHA the PCR product and the excised sCD sequence into the linearised vector pTREAutoR3, subsequent cloning steps were performed as described in chapter 2.2. Successful incorporation of the constructs into pTRE vector were identified by restriction digest using BamHI and NdeI. Subsequently, DNA sequences of positive clones were verified by sequencing. Figure 4-4B shows the successful generation of Dox-inducible lentiviral expression constructs for sCD, as demonstrated by the replacement of GFP with the sCD and sCDHA fragments.

4.4.2 Analysis of sCD Expression and secretion using the inducible lentiviral constructs

Following generation of the inducible lentiviral sCD expression vectors, a lentiviral virus stock was generated for pTREsCD and pTREsCDHA constructs and used to transduce HEK293 cells. To determine the expression and secretion of the 5-FC-converting enzyme CD in the transduced cells, Western blot analysis of the cell lysates and concentrated cell culture supernatants was performed (Figure 4-4C). The same amount of protein for the lysates (30 µg/well) and the same volume for supernatants (30 µL/well) were loaded into each well of the 10% SDS polyacrylamide gel and the detection was performed with α-HA antibody (1:1000). The results showed that following Dox-induction

the HA-tagged enzyme was expressed and secreted by the transduced cells. However, a low level of transcription took place even in the absence of the inducer, as a weak band was detected in supernatants of untreated sCDHA-transduced HEK293 cells. However, given that expression of the enzyme was very low, it would not be expected to lead to an immune response against CD.

Furthermore, an immunofluorescence assay was used to confirm the expression of HA-tagged sCD in pTREsCDHA-transduced HEK293 cells. Non-transduced, pTREAutoR3- and pTREsCD-transduced HEK293 cells were used as a control. To this end, $1x10^4$ cells were seeded into wells of a 6-well plate and cultured for three days. All cell lines used for this experiment were cultured in the absence or presence of 2 mg/ml Dox. Cells were fixed and the expression of the HA-tagged protein was visualised by performing the IF staining protocol as described in 2.5.1. The pTREAutoR3-transduced HEK293 cells were used to confirm that the concentration of Dox used in this experiment was sufficient to induce Dox-dependent expression. As shown in Figure 4-4D, GFP expression of pTREAutoR3-transduced HEK293 cells was induced in the presence of Dox. Furthermore, the HA-tag was detected only in pTREsCDHA-transduced HEK293 cells following addition of Dox.



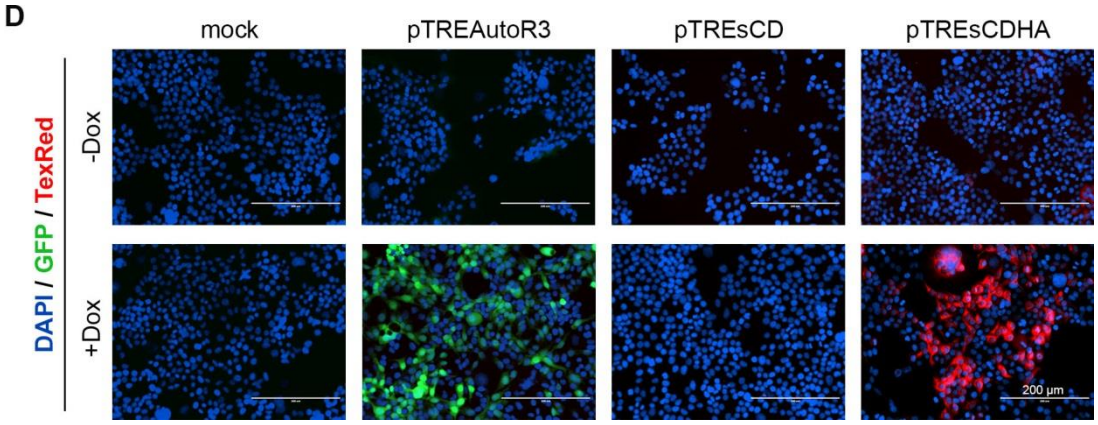


Figure 4-4: Generation of inducible lentiviral expression constructs for sCD and analysis of sCD expression/secretion. (A) Illustration of cloning strategy for the generation of pTREsCD and pTREsCDHA (TRE=Tet-responsive element, IRES=internal ribosome entry site, rtTA=reverse transcriptional activator)**. (B)** Lentiviral Dox-inducible expression vector pTREAutoR3, pTREsCD and pTREsCDHA were cut with restriction enzyme EcoRI. This demonstrated, that the sequence for eGFP (846 bp) was replaced by sCD (1388 bp) or sCDHA (1451 bp) sequence, respectively. **(C and D)** Analysis of sCD expression and secretion in pTREsCD- and pTREsCDHA-transduced HEK293 cells by Western blot (C) and IF (D) using an α-HA antibody.

4.4.3 Assessment of *in vitro* functionality of the inducible lentiviral sCD expression construct

Thus far, only the expression of sCD in pTREsCD and pTREsCDHA transduced cells had been tested and the functionality of the expressed enzymes still needed to be examined. Therefore, a secretion assay was performed in which FLuc-expressing cancer cells (EO771 and PyMT) were cultured with CM collected from sCD- or sCDHA-transduced HEK293 cells that were cultured with 1 µM Dox for four days. Viability of cancer cells was measured indirectly via bioluminescence imaging following addition of luciferin. The medium was changed daily, followed by addition of 0.1 mM 5-FC and 7.5 µg luciferin to the cells 10 min prior to imaging. The results of this experiment are displayed in Figure 4-5A and B. A significant reduction in bioluminescence signal and, thus, reduced viability was observed in EO771 and PyMT cells following the combined addition of CM and 5-FC. Thus, demonstrating that pTREsCD and pTREsCDHA-transduced HEK293 cells expressed and secreted a functional enzyme into the medium after Dox induction. The secreted enzyme was capable of converting 5-FC to the toxic compound 5-FU which led to the death of cancer cells.

Taken together these results confirm the *in vitro* functionality of the Dox-inducible lentiviral expression constructs pTREsCD and pTREsCDHA. HEK293 cells produced

and secreted the functional enzyme sCD, following lentiviral transduction with pTREsCD or pTREsCDHA. These generated constructs were then applied as part of the cell therapy approach to treat brain metastases in *in vivo* models.

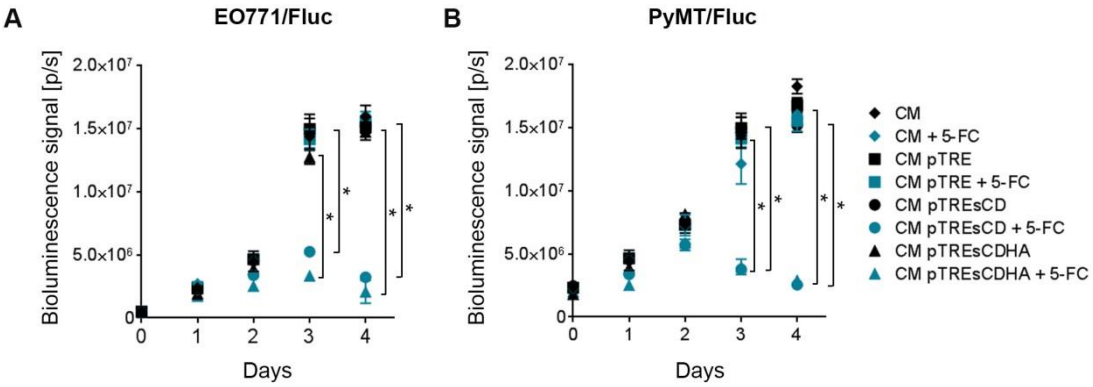
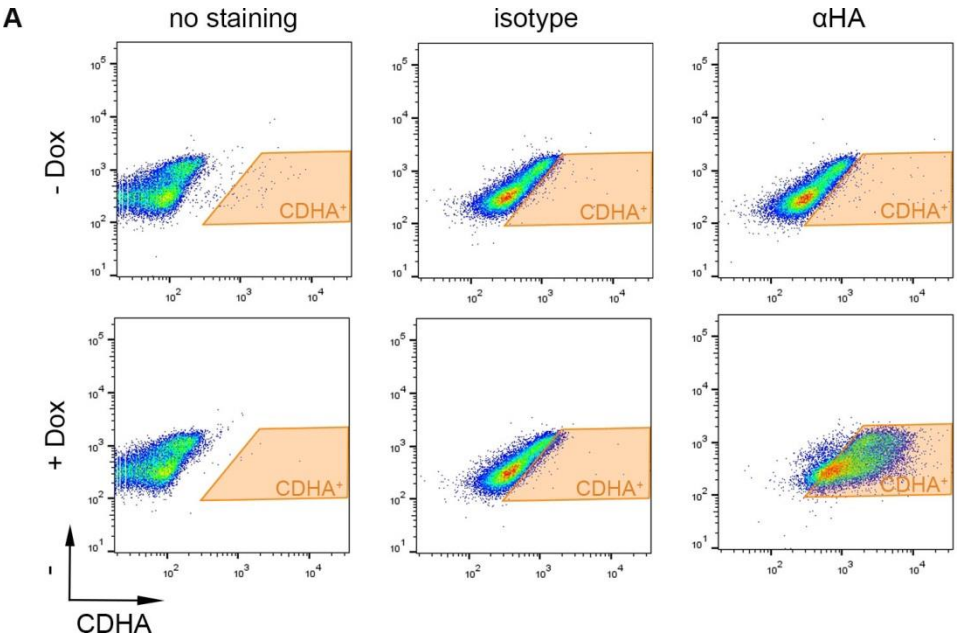


Figure 4-5: Assessment of *in vitro* functionality of lentiviral expression constructs for CD. (A and B) FLuc expressing cancer cells (EO771 and PyMT) were cultured with CM collected from sCD or sCDHA-transduced HEK293 cells that have been induced with 1 µM Dox for 4 days. Bioluminescence signal of cancer cells was measured after addition of luciferin to the medium daily. The sCD and sCDHA transduced HEK293 cells cultured in the presence of Dox secreted sCD into culture medium, which led to a reduced bioluminescence signal of CM-cultured cancer cells after 0.1 mM 5-FC administration (n=3 technical replicates, mean+/-SEM). Two repeats of the experiment were performed of which one is shown here. Statistical significance determined with multiple t-tests using the Holm-Sidak method, with alpha=5%.

4.4.4 Analysis of inducible sCDHA expression *in vivo*



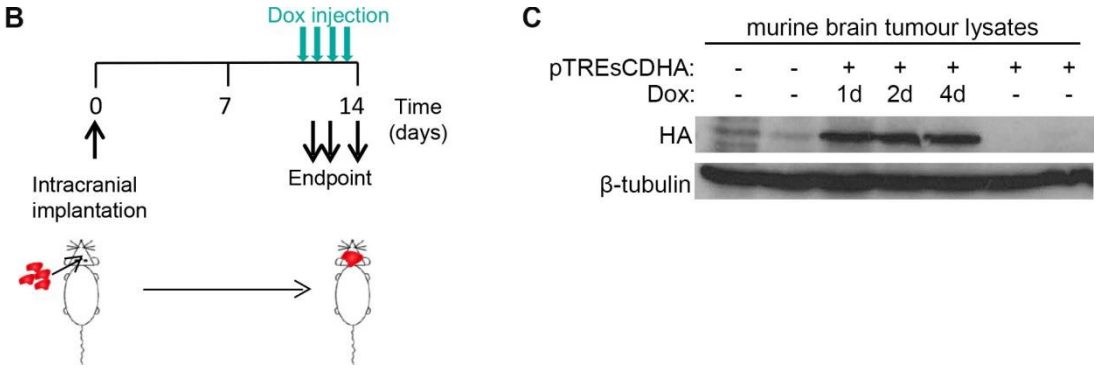


Figure 4-6: Analysis of inducible sCDHA expression *in vivo*. (A) Expression of HA-tagged CD in pTREsCDHA transduced cancer cell line EO771 was confirmed by flow cytometry. **(B)** Experimental outline: C57BL6/J mice received intracranial implantation of $1x10^5$ EO771/pTREsCDHA cells. Daily ip administration of 2 mg doxycycline was performed from day 10 post cancer cell implantation. **(C)** *In vivo* expression of HA-tagged sCD following daily ip administration of 2 mg Dox was confirmed by Western Blot analysis of brain tumour lysates after one, two or four days of Dox administration using α-HA antibody.

To assess the *in vivo* functionality of the inducible lentiviral expression construct pTREsCDHA, it was first tested if the expression of sCD can be induced in transduced tumour cells located in the murine brain following systemic administration of Dox. It is known that Dox can cross the BBB (Agwuh and MacGowan, 2006) and has been previously used in a gene therapy regime to target a CNS disease (Wilsey et al., 2002).

Firstly, the murine breast cancer cell line EO771 was transduced with the pTREsCDHA virus at an MOI of 100. The titre of the virus stock was determined by using the reverse transcriptase assay. Three days after the transduction the cells were treated with 2 µg/ml Dox for two days and then analysed for sCDHA expression. The expression of the HA-tagged protein in the cells was analysed quantitatively by flow cytometry using an intracellular staining using an α-HA antibody. The results of this analysis are displayed in Figure 4-6A. Approximately 90% of the transduced cell line expressed sCDHA following Dox induction. Subsequently, the cell line EO771/sCHDA was used for intracranial tumour implantation (Figure 4-6B). For this experiment five mice received an intracranial implantation of $1x10^5$ EO771/pTREsCDHA cancer cells and two mice received $1x10^5$ EO771 cancer cells. Dox (2 mg) was administered for one, two or four days before the endpoint by daily ip injections. At the end of the experiment, brain tumour tissue was isolated and prepared for Western blot analysis. This analysis showed an induction of expression of sCDHA within the brain tumour tissue after systemic administration of Dox (Figure 4-6C). Moreover, expression was observed after 1 day of doxycycline administration indicating a rapid response. Based on this result a further *in*

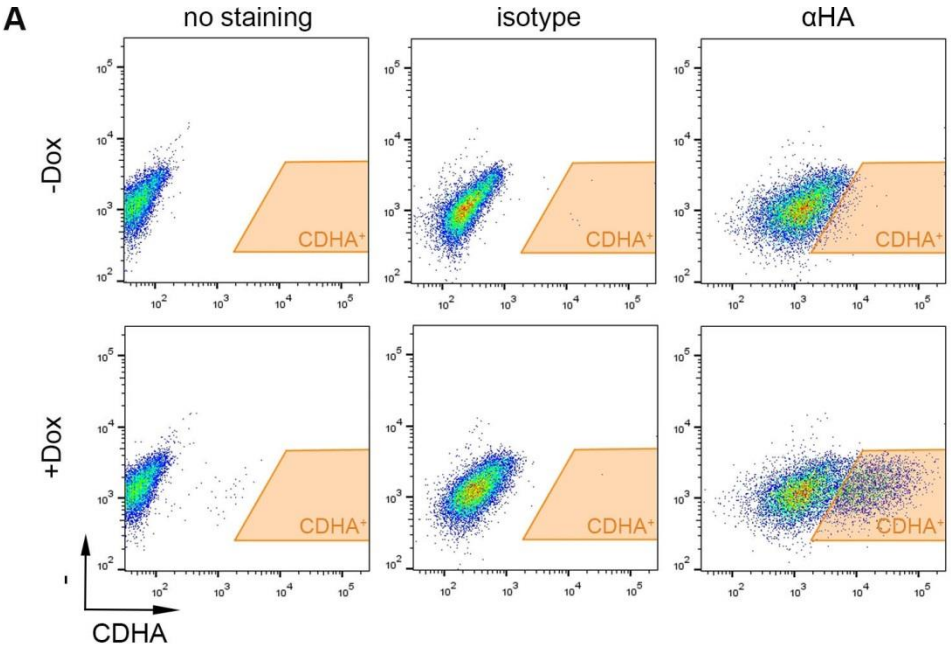
vivo experiment was performed to test the functionality of this inducible enzyme prodrug therapy.

4.4.5 Testing the functionality of the inducible enzyme prodrug approach *in vivo*

In order to test the *in vivo* functionality of the inducible lentiviral expression construct in a brain tumour model, a new sCDHA expressing EO771 cell line was generated. To this end, the cell line EO771/FLuc was transduced with the pTREsCDHA virus at MOI 10 and the expression of sCDHA in this transduced cell line was then analysed by flow cytometry. To this end, an intracellular staining of the transduced cell line, treated with 2 µg/ml Dox for two days, was performed. This experiment revealed that approximately 25% of the transduced cell line expressed the enzyme (Figure 4-7A). This ratio of one sCDHA expressing cell to three cancer cells within the brain tumour environment recapitulates the ratio of infiltrating bm-derived myeloid cells to cancer cells within brain metastases (chapter 3.5). To analyse whether this ratio was sufficient to achieve a therapeutic effect, this cell line was used for subsequent implantation of $1x10^5$ cancer cells in the brain striatum of 16 mice (Figure 4-7B). The FLuc expression of the cancer cells enabled the non-invasive measurement of the brain tumour growth over time via bioluminescent imaging following ip injections of 1.2 mg luciferin. The brain tumour growth of these 16 mice is shown in Figure 4-7C. Six days after the implantation, the mice were split into two groups based on average distribution of bioluminescence tumour signals. One group received daily ip injections of 800 µL PBS, whereas the other mice received daily injections of 500 mg/kg 5-FC from day seven post implantation. Furthermore, all mice received daily ip injections of 2 mg Dox six days after the intracranial implantation. After three days of treatment a reduced tumour growth was observed in mice that had received 5-FC compared with PBS-treated mice. This difference in tumour growth was increased over time. However, due to neurological symptoms (experimental endpoint) caused by the brain tumour growth, all mice in the control group and half of the mice in the treatment group had to be culled on day 13. Only 5-FC treated mice that did not show any neurological symptoms remained and the treatment in these mice was continued.

The difference in brain tumour signals between days six and 13 post implantation between the two treatment groups revealed a significant decrease, suggesting a significantly lower tumour burden in the treatment group (Figure 4-7D). Moreover, the 5-FC treatment appeared to retard tumour growth, whereas tumours in the PBS-treated

group continued to increase in size. Furthermore, Kaplan-Meyer analysis of this experiment showed that the 5-FC treated mice had a significant survival advantage over the control group (Figure 4-7E). The experiment was terminated as soon as a significant difference in survival had been achieved with two healthy mice still remaining in the treatment group. The median survival time of PBS-treated mice was 13 days, compared with 13.5 days for 5-FC treated mice. These data show that the induction of sCDHA expression in cancer cells used in combination with 5-FC leads to reduced tumour growth when the sCD carrier cells are present at a ratio of 1:3.



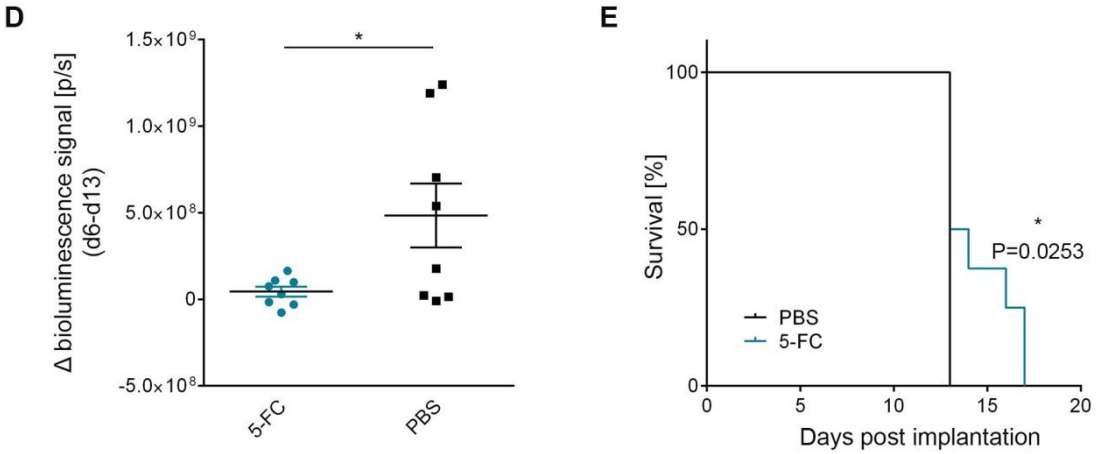


Figure 4-7: Induction of sCDHA expression in cancer cells in combination with 5-FC results in reduced tumour growth *in vivo*. (A) Expression of HA-tagged sCD in pTREsCDHA-transduced cancer cell line EO771/FLuc was confirmed by flow cytometry. **(B)** Experimental outline: C57BL6/J mice received intracranial implantation of $1x10^5$ EO771/FLuc/pTREsCDHA cells. Daily ip administration of 2 mg doxycycline and 500 mg/kg 5-FC or PBS (control group) was performed from day six and seven post cancer cell implantation, respectively. **(C)** Brain tumour growth was measured by bioluminescence imaging (n=8 mice per group, mean +/-SEM). **(D)** Further analysis of bioluminescence signals was performed to show the growth difference of brain tumours in mice that received 5-FC versus those that received PBS between day six and 13 post cancer cell implantation (n=8 mice per group, Δ for every mouse d6-d13 and mean of Δ +/-SEM for both groups is shown). Statistical significance was determined using a two-tailed unpaired t-test, p=0.0332. **(E)** Kaplan-Meier plot shows overall survival of 5-FC treated and non-treated mice (n=8 mice each), statistical significance was determined with Log-rank (Mantel-Cox) test, p=0.0253.

4.5 Discussion

After the tumour-homing capability of the progeny of systemically administered HSCs was established and their suitability to act as delivery vehicles of therapeutic molecules to target brain metastases was demonstrated in chapter 3, the choice of therapy was addressed next. The use of an enzyme prodrug therapy was selected to localise the toxic effect of the delivered therapeutic molecule at the tumour site, since the therapy would only be induced by the presence of the prodrug. Recent publications demonstrated the use of genetically engineered stem cells as cellular vehicles, thereby delivering the enzyme prodrug therapy CD/5-FC to treat brain tumours. An MSC-mediated CD/5-FC therapy showed a therapeutic effect in rat glioma (Fei et al., 2012, Kosaka et al., 2012). Further, the CD/5-FC therapy has also demonstrated therapeutic efficacy in melanoma brain metastases, medulloblastoma and glioma when delivered by NSCs (Aboody et al., 2006, Kim et al., 2006, Aboody et al., 2013). Moreover, the chemotherapeutic agent 5-FU is well established as a treatment option for breast cancer patients (Cameron et al., 1994). Based on these findings, the enzyme prodrug therapy CD/5-FC was selected for

use in the HSC-based cell therapy regime. To this end, a lentiviral expression construct of the prodrug converting enzyme cytosine deaminase was generated and its *in vitro* functionality assessed. However, in the first instance the cell-based therapy exhibited no therapeutic effect on syngeneic brain tumours. Subsequent analysis of sCD expression in leukocytes isolated from the mice that have received pFUWsCD-transduced HSCs revealed extremely low expression levels of CD compared with freshly transduced HSCs. The expression level of the enzyme in TAMs was too low to convert a sufficient amount of 5-FC into 5-FU and therefore did not induce a detectable level of tumour cell death. Low sCD expression in mice leukocytes may be due to the immunogenic potential of HSC progeny which express high amounts of the bacterial enzyme. Therefore, it is possible that only leukocytes expressing low levels of sCD could avoid the immune response and remain in the mice. However, this effect has not been described by others who used the CD/5-FC approach in a cell based therapy employing cell vehicles other than HSCs (Aboody et al., 2006, Kim et al., 2006, Fei et al., 2012, Kosaka et al., 2012, Aboody et al., 2013). This difference could be due to several factors such as, lack of systemic administration and a shorter time period at which the stem cells were present in the mice.

Another explanation could be that the transduction efficiency of the pFUWsCD in the HSCs was too low. The titration of the generated pFUWsCD virus stock was performed using the Roche reverse transcriptase assay, which detects reverse transcriptase and correlates this to a standard virus stock with a known concentration. The standard virus stock used in this case was a pFUGW virus and the titre was determined by flow cytometric analysis of HEK293 cells transduced with different amounts of the virus stock. As this method only indirectly leads to the determination of the virus titre it strongly depends on the standard virus at a known concentration. The pFUGW transduction efficiency of HSCs that were used for bm engraftment in mice was also low compared with published literature, as mentioned in chapter 3.6. However, a better method to determine the virus titre could be the Lenti-X provirus quantitation kit that detects viral integration sites in the genome of a lentiviral transduced cell and would be a direct readout for transduction efficiency.

To circumvent the possible immunogenicity of CD expressing leukocytes, a lentiviral inducible expression system for CD was generated and its *in vitro* functionality tested. The efficacy of this CD/5-FC therapy was determined in a syngeneic brain metastases model by performing an intracranial implantation with the cell line EO771/FLuc/sCDHA. Only 25% of this cell line expressed the enzyme following doxycycline induction. This ratio of one sCDHA expressing cell to three cancer cells within the brain tumour following

implantation recapitulated the ratio of infiltrating bm-derived myeloid cells to cancer cells within brain metastases. The mice that received the 5-FC treatment exhibited a slowed tumour growth and a prolonged survival compared with the control-treated mice. These promising results need to be confirmed in an HSC-based therapy in syngeneic brain metastases models.

Another way of modifying the therapy to prevent the possible immunogenic potential of CD expressing leukocytes is to use an alternative promoter that is specifically up-regulated in brain metastases-infiltrating myeloid cells. This would reduce the potential of systemic toxicities of this HSC-based therapy, as HSC progeny naturally accumulate in organs like the spleen, lungs, liver, blood and bone marrow. The identification of such TAM-specific promoters is addressed in chapter 5 of this study.

The genetic modification of the HSCs for this study was performed using lentiviral transduction. By contrast to other retroviruses (Miller et al., 1990), lentiviral vectors can genetically engineer non-dividing HSCs (Reiser et al., 1996). During this process, lentiviral vector DNA is integrated into the HSC genome at random positions which may cause mutagenesis (Hacein-Bey-Abina et al., 2003). However, recent findings suggest that the integration of viral DNA is not random but directed to specific active genes and related to genome organisation (Marini et al., 2015). The risk of mutagenesis by retrovirally mediated gene transfer has previously been considered to be very low in humans (Stocking et al., 1993). The transduction efficiency achieved in this study is low compared with published data and is a likely reason for the lack of therapeutic effect observed in the HSC-based therapy in experimental brain metastases models. It is known that an increase in MOI correlates with a higher copy number in target cells (Kustikova et al., 2003, Li et al., 2003). However, to minimise the risk of insertional mutagenesis a low MOI is favoured in HSCs for clinical trial protocols. There are established protocols for lentiviral transduction using a low MOI in the literature (Zielske and Gerson, 2002, Kurre et al., 2004). However, HSC transduction efficiencies are too low and given that lower MOIs are favoured in clinical trials an alternative method to genetically modify HSCs needs to be considered. The CRISPR/Cas9 (Jinek et al., 2012, Cong et al., 2013, Mali et al., 2013) system may represent a possible option in the future.

Furthermore, the enzyme prodrug therapy CD/5-FC used in this study can be optimised in various ways to enhance the therapeutic effect on brain metastases. The bacterial enzyme (bCD) could be replaced by the yeast CD (yCD) sequence, as it has been shown that yeast CD is superior to its bacterial counterpart in 5-FC conversion (Kievit et al., 1999). Kievit and colleagues demonstrated that HT29 xenograft-bearing nude mice showed complete tumour regression in response to yCD/5-FC treatment, whereas no

tumours treated with bCD/5-FC were cured. Further, the enzyme could be fused to uracil phosphoribosyltransferase (UPRT) which has been shown to enhance the sensitivity of cells to 5-FU by accelerating the ability of these cells to metabolise 5-FU to its active metabolites (Erbs et al., 2000). A dual suicide gene therapy could also display a possible option for modification to enhance the therapeutic effect of the CD/5-FC therapy. Another widely used enzyme prodrug approach involves herpes simplex virus thymidine kinase (TK) in combination with ganciclovir (GCV). Previously, the combined suicide gene therapy CD-TK delivered by NSCs led to a prolonged survival of mice with brain metastases (Wang et al., 2012). This double gene therapy mediated by NSC has also demonstrated therapeutic efficacy in glioblastoma models (Niu et al., 2013, Lee et al., 2014).

For the HSC-based cell therapy, the HSCs can be engineered to deliver a variety of anti-cancer agents, including prodrug-activating enzymes, apoptosis-inducing agents, interleukins, and antibodies. The sensitivity of the breast cancer cell lines PyMT and EO771 to other enzyme prodrug therapies, i.e. thymidine kinase/ganciclovir or carboxylesterase/irinotecan, could also be tested. Another therapeutic molecule that could be delivered by HSCs and their progeny to target brain metastases is tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL induces apoptosis in tumour cells (Pitti et al., 1996), whilst sparing most normal cells (Ashkenazi et al., 1999). Engineered NSCs expressing TRAIL have shown efficacy in rodent glioma models, demonstrating an increase in apoptotic cells in these tumours, and a significant inhibition in the rate of tumour progression (Ehtesham et al., 2002a). A different therapeutic approach that could be applied in HSC-based cell therapy is the use of interleukins. Most interleukins mediate cell signalling between various cells of the immune system. To restrict HSC-delivered interleukin signalling to the brain metastases region, the expression of the interleukins would have to be regulated by specific promoters that induce expression only in the brain metastases-infiltrating myeloid cells. Benedetti and colleagues showed the therapeutic efficacy of a neural progenitor cell-based delivery of IL-4 to syngeneic mouse glioma models (Benedetti et al., 2000), thereby utilising the IL-4 initiated T-cell mediated immune response to cancer cells. The anti-tumour potential of IL-12 (Tugues et al., 2015) has also been explored amongst others in a NSC-based therapy to treat glioma (Ehtesham et al., 2002b) and in a MSC-based therapy to treat breast cancer (Eliopoulos et al., 2008).

Overall, HSCs can be engineered to express various anti-tumour gene products to target brain metastases. However, many factors need to be considered when choosing a therapeutic gene product: the level and duration of expression of the therapeutic gene at

tumour site, the appropriateness of the molecular target, and the ability of the therapeutic entities to interact with the cancer cells.

5. Developing a strategy for improved specificity of HSC-based cell therapy targeting brain metastases

5.1 Introduction

HSC therapies have been successfully used in the treatment of malignant haematologic diseases. Additionally, genetic diseases of the blood, such as immunodeficiencies, thalassemia (Cavazzana-Calvo et al., 2010) or sickle cell anaemia (Shenoy, 2011), have been corrected by HSC therapies. Further, HSC therapy can also correct some genetic diseases where a secreted enzyme, normally expressed in haematopoietic lineages, can be taken up by surrounding affected cells, which includes the neurodegenerative lysosomal storage disease (LSD) Mucopolysaccharidosis (MPS) I Hurler (Wynn et al., 2009). So far, only one preclinical study in breast cancer has demonstrated therapeutic efficacy of HSC gene therapy (Li et al., 2009). In order to develop an HSC-based cell therapy to treat brain metastases the biodistribution of injected HSCs and their progeny needed to be considered. HSCs give rise to haematopoietic cells of myeloid and lymphoid lineages and these are known to home to different organs, sites of inflammation and tumours. Therefore, once genetically engineered HSCs expressing a therapeutic agent are used in this cell-based therapy, the agent could also accumulate in other organs and therefore potentially lead to systemic toxicities. To circumvent this issue, a goal of this study was to identify promoters of genes that are specifically up-regulated in brain metastases-infiltrating myeloid cells. These could then be used to drive the brain metastases-specific expression of therapeutic molecules within myeloid cells and restrict the delivery of therapeutic agents to brain lesions.

The use of cell type or tissue-specific promoters for gene therapy has already been explored by other research groups. Hatzoglou et al. demonstrated liver-specific expression of different genes under the control of the phosphoenolpyruvate carboxykinase promoter (Hatzoglou et al., 1990). Directing toxicity toward B-cells was achieved by the transcriptional expression of diphtheria toxin A-fragment controlled by immunoglobulin-kappa promoter and enhancer (Maxwell et al., 1991). Vile and Hart (1993) explored the use of the tyrosinase promoter to direct gene expression to melanoma cells. Hepatoma cell-specific expression for gene therapy was achieved by utilising the alpha-fetoprotein promoter (Arbuthnot et al., 1996). Further cell and tissue-specific promoters have been explored for gene therapy: the mucin-1 promoter for mammary carcinoma (Chen et al., 1995), the prostate-specific antigen promoter for prostate carcinoma (Lee et al., 1996), or the neuron-specific enolase promoter for neuronal cells (Abdallah et al., 1996). Other studies have used transcriptional control elements, that can stimulate transcription in response to disease-specific alterations, such as hypoxic conditions (Dachs et al., 1997) or loss of cell cycle checkpoints in tumours (Parr et al., 1997).

Interestingly, a transgenic mouse model has been developed that uses the myeloid specific CD11b promoter to drive the expression of thymidine kinase (Gowing et al., 2006). Thereby, myeloid cells can be depleted following administration of the prodrug ganciclovir. However, this promoter does not allow differentiation between macrophages and myeloid cells and is therefore not appropriate for this study.

5.2 Aims of this chapter

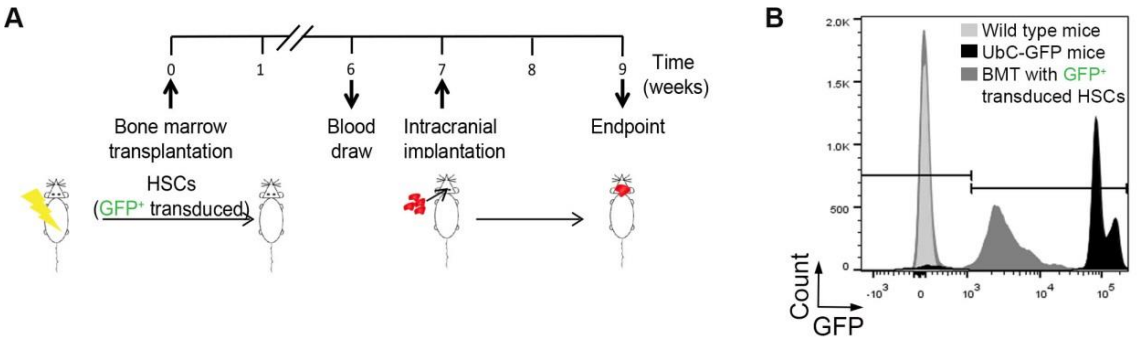
In order to circumvent potential side effects of the HSC-based therapy, promoters specifically active in brain metastases-infiltrating myeloid cells can be used to drive the expression of therapeutic agents. Therefore, the aims of this chapter are:

- To identify genes specifically up-regulated in brain metastases-infiltrating myeloid cells by genome-wide gene expression analysis of $CD11b^+$ cells isolated from brain metastases, bone marrow and the spleen of chimeric mice with GFP^+ bone marrow.
- To validate the specific expression patterns of identified genes in human and murine brain metastases samples.
- To validate promoter-reporter constructs *in vitro* and *in vivo* using promoters of specifically up-regulated genes in brain metastases-infiltrating TAMs.

5.3 Biodistribution of systemically administered HSCs and their progeny in mice

HSCs give rise to haematopoietic cells of myeloid and lymphoid lineages and these cells can home to different organs, sites of inflammation and tumours. Thus, the therapeutic agents introduced by HSCs in the cell based therapy approach would home to brain metastases and other tissues. To determine the extent of this potential non-specific therapeutic delivery, the amount of the GFP^+ HSC progeny in different organs of brain metastases-bearing chimeric mice with GFP-tagged bone marrow was determined. To generate chimeric mice, the bone marrow in C57BL/6J mice (n=3) was ablated by whole body irradiation, followed by the transplantation of HSCs that have been lentivirally transduced with pFUGW at an MOI of 30 (experimental outline is shown in Figure 5-1A). Six weeks after the BMT, the blood was analysed by flow cytometry to determine if the bone marrow was successfully reconstituted by transplanted HSCs (Figure 5-1B). About

35-60% of all leukocytes in chimeric mice displayed GFP expression, compared with naïve (0.1%) and UbC-GFP mice (91%). Subsequently, different organs were dissociated and analysed by flow cytometry (Figure 5-1C). Up to 30% of $CD45^+$ haematopoietic cells in these organs expressed GFP. The presence of GFP^+ cells could be detected in blood (45%), bone marrow (25%), spleen (20%), lungs (20%) and liver (10%) as shown in Figure 5-1D. These findings confirmed that the progeny of GFP-tagged HSCs infiltrate brain metastases (as shown in chapter 3), as well as other tissues including the bone marrow, spleen, lungs and liver.



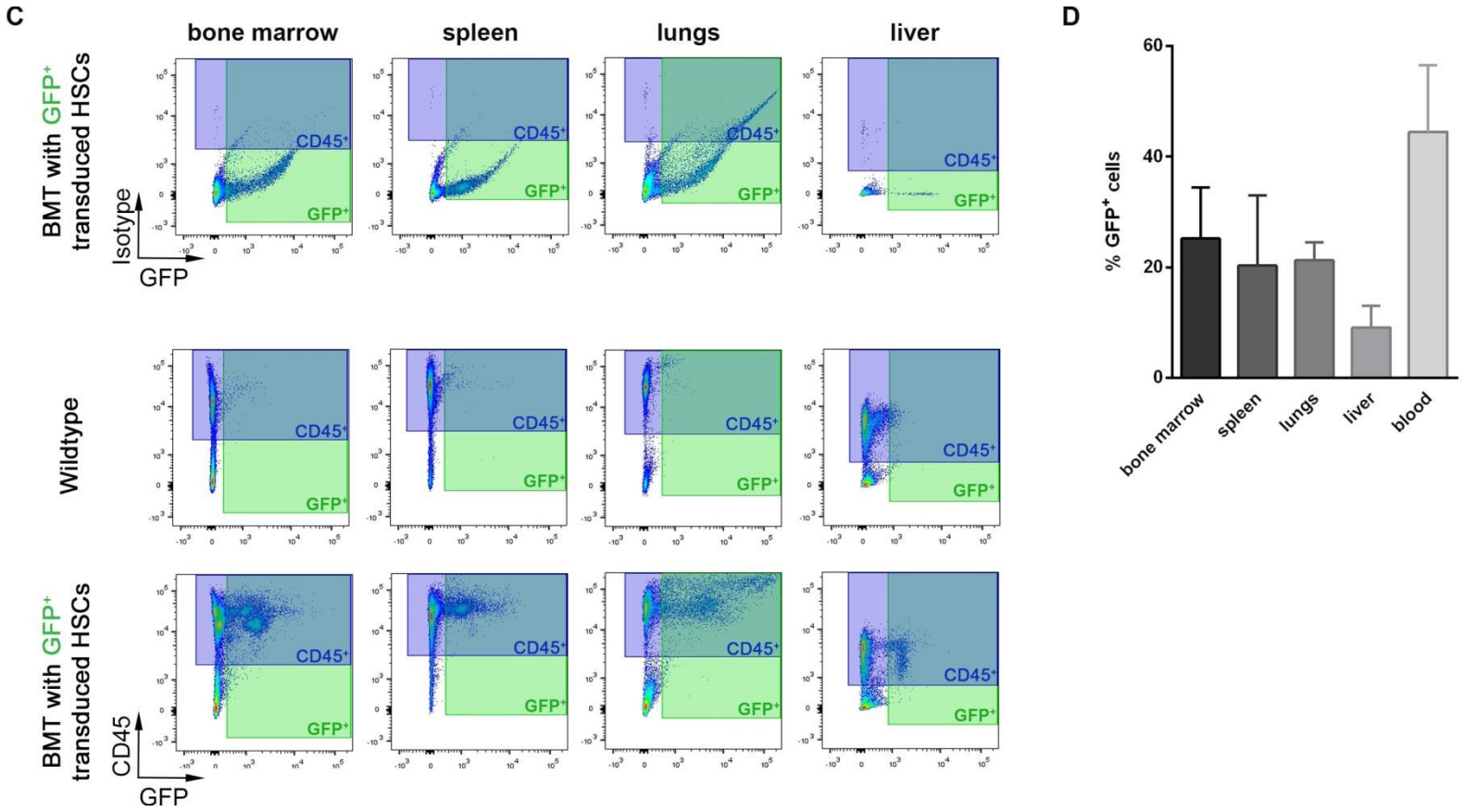


Figure 5-1: Distribution of bone marrow progeny in mice that received engraftment of GFP transduced HSCs. (A) Mice underwent bone marrow engraftment with GFP transduced HSCs. **(B)** The blood of the mice was analysed by flow cytometry to check reconstitution six weeks after BMT. **(C)** The progeny of GFP transduced HSCs in different tissues of mice six weeks after BMT was analysed by flow cytometry. **(D)** The quantification of the flow cytometry analysis of different organs shows the percentage of GFP^+ cells of the mice that received the engraftment (n=3 technical replicates, mean+SEM is shown).

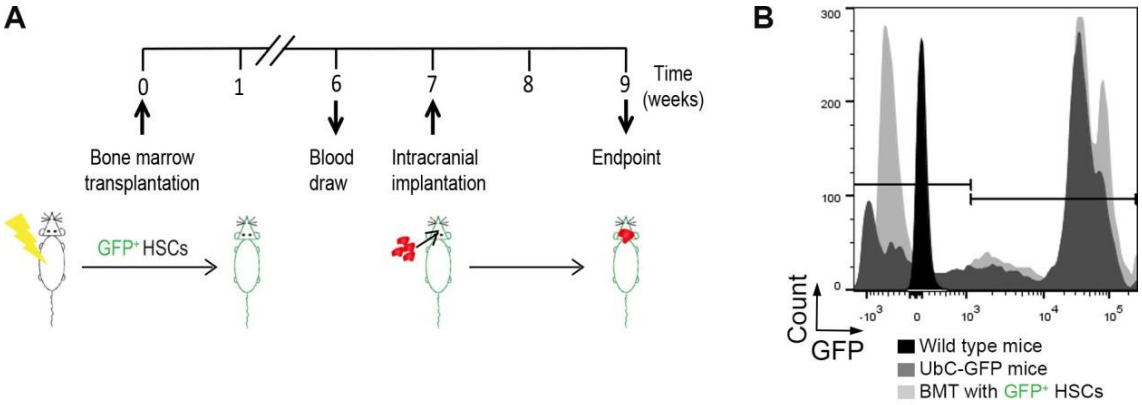
5.4 Comparative gene expression analysis of myeloid cells isolated from different tissues: Identifying TAM-specific genes

To restrict the delivery of therapeutic agents exclusively to brain metastases, and thereby limit potential systemic toxicities, gene promoters specifically up-regulated in brain metastases-infiltrating myeloid cells could be used to drive the brain metastases-specific expression of therapeutic molecules. To identify such gene promoters, the following *in vivo* experiment was performed (Figure 5-2A). Firstly, chimeric mice with GFP-tagged bone marrow were generated. To this end, the bone marrow in C57BL/6J mice was ablated by whole body irradiation, followed by the transplantation of HSCs isolated from transgenic C57BL/6J UBC-GFP mice which express GFP under the control of the human ubiquitin C promoter in all tissues. Six weeks after the BMT, blood was drawn from the tail vein of the mice and analysed by flow cytometry (Figure 5-2B). This was done to determine if the bone marrow was successfully reconstituted by the transplanted HSCs. About 78-92% of all leukocytes in chimeric mice expressed GFP, compared with wild type (0.24%) and UbC-GFP mice (92%). These chimeric mice received intracranial injections of either $1x10^5$ EO771 breast cancer cells or $1x10^5$ PyMT breast cancer cells or PBS alone. After the injected cancer cells have formed large brain lesions, GFP^+CD11b^+ cells were isolated from brain metastases by FACS (Figure 5-2C) and $CD11b^+$ cells from the bone marrow and spleen by MACS. The total RNA of the isolated myeloid cells was extracted and subjected to genome-wide gene expression analysis using the Illumina BeadArray system.

To identify genes up-regulated in brain metastases across both cancer cell models, the gene expression data was divided into two groups: the spleen/bone marrow group and the brain metastases group. A total of 5972 probes were identified as being significantly differentially expressed (FDR < 1%) between these two groups. The heatmap shown in Figure 5-2D displays the top 150 differentially expressed probes that have been subjected to hierarchical clustering. The fold changes of probes that were up-regulated in brain metastases were larger than 10 for 119 probes and even larger than 100 for 9 probes. Among the top up-regulated genes were matrix metalloproteinases (MMP13, MMP14), chemokines and their receptors (CCL7, CXCL10, CCL2, CCR5) and genes associated with anti-inflammatory phenotype (Arg1) as well as macrophage differentiation/activation (disabled homologue 2 (Dab2), Trem2). To find probes that were up-regulated in brain metastases samples compared with spleen/bone marrow samples across both cancer models individually, differential gene expression analysis was performed (Figure 5-2E). Some up-regulated probes varied strongly between

EO771 and PyMT model, while other probes displayed comparable fold changes. For the identification of robustly activated gene promoters further analysis focussed on 12 genes that were highly up-regulated across different cancer cell models. Other criteria used to select the candidate gene promoters included the existence of the human homologue and the specific expression patterns of the human homologue.

For example, the murine Ms4a6d gene was strongly up-regulated in bm-derived myeloid cells that were infiltrating brain metastases. This gene is predicted to be the homologue of the human MS4A6A and MS4A6E genes. These human genes are reciprocally homologous to the other members of the murine Ms4s6 family (Ms4a6b, Ms4a6c and Ms4a6d) according to HUGO gene nomenclature committee (HGNC) (genenames.org, 2015). However, the other members of the murine Ms4s6 family were not found to be strongly up-regulated within the brain tumour-infiltrating myeloid cell population. Further, the human gene MS4A6A exhibits a basal expression level in spleen, bone marrow, liver, monocytes and B-cells (Genatlas, 2004). Based on the imprecise orthology of this murine gene and the non-specific expression of its predicted human homologue, this candidate gene was excluded from further validation analysis. Based on high fold changes in the EO771 and PyMT model, another interesting gene candidate was Fcrls. However, this candidate was also excluded from further analysis, as there is no human orthologue (genenames.org, 2015). Additional candidates, i.e. Olfml3, Slamf9, Rgs, MMP13, Ccl3 and Gp38, that were specifically up-regulated in $CD11b^{+}GFP^{+}$ cells in brain tumours versus spleen/bone marrow across both models, were also removed from further analysis because their fold changes were either too low or differed strongly between the two models.



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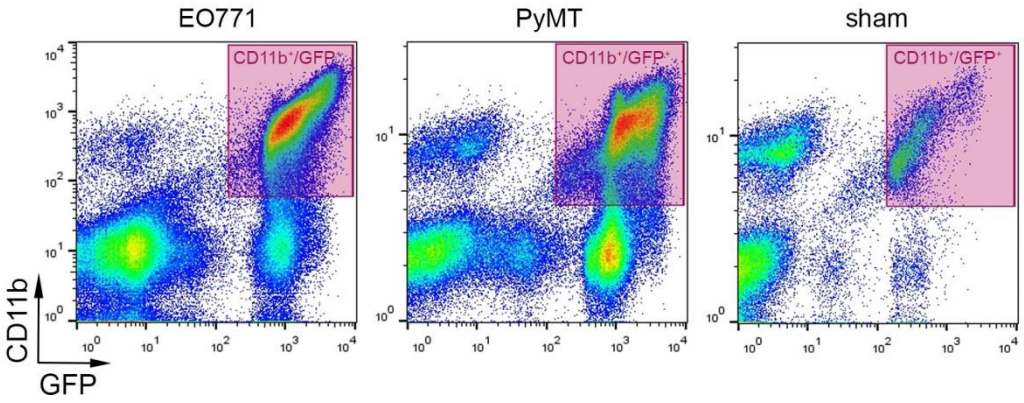
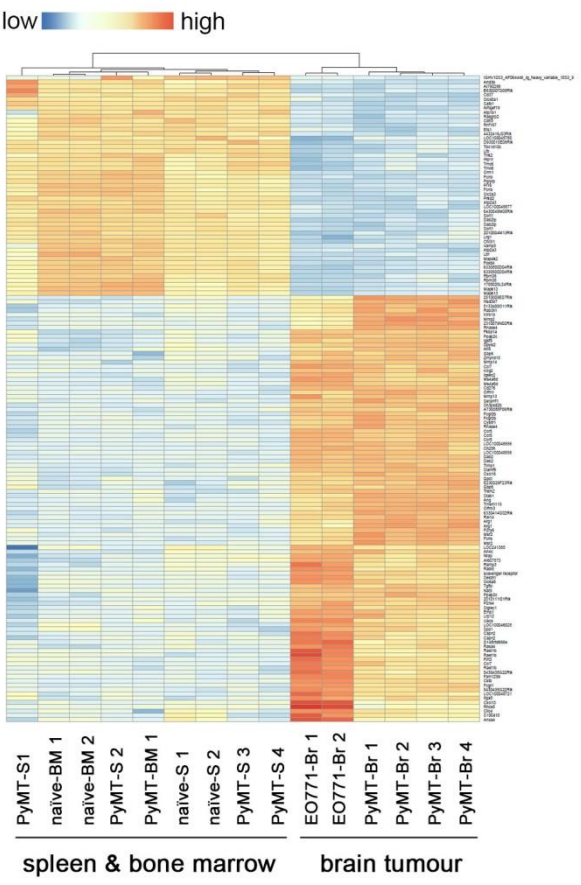

EO771
PyMT
sham
CD11b+/GFP+
CD11b+/GFP+
CD11b+/GFP+
CD11b
GFP

D


low
high
PyMT-S1
naïve-BM 1
naïve-BM 2
PyMT-S 2
PyMT-BM 1
naïve-S 1
naïve-S 2
PyMT-S 3
PyMT-S 4
EO771-Br 1
EO771-Br 2
PyMT-Br 1
PyMT-Br 2
PyMT-Br 3
PyMT-Br 4
spleen & bone marrow
brain tumour

E

	Fold change			
	EO771		PyMT	
	Brain vs. spleen	Brain vs. bm	Brain vs. spleen	Brain vs. bm
Dab2	38.05	44.67	29.83	25.43
Mmp14	38.95	37.80	32.01	23.41
Ccr5	21.03	35.15	15.72	17.52
Ms4a6d	22.33	33.95	14.00	16.55
Spp1	62.18	29.31	13.40	13.31
Ecm1	24.61	26.40	9.92	11.3
Fcrls	24.33	22.53	63.19	58.91
Trem2	17.57	12.32	15.37	7.99
Emp1	89.72	78.16	13.47	10.16
Ccl7	70.49	66.88	12.94	11.83
Gpnmb	116.08	64.84	11.73	8.21
Cxcl10	56.13	53.77	9.24	9.53
Arg1	7.21	6.61	57.07	54.13
Cxcl16	9.41	20.45	20.46	26.67
Olfml3	7.42	7.25	17.80	17.34
Slamf9	10.10	15.81	14.65	12.12
Rgs1	17.99	61.98	7.68	12.69
Mmp13	24.31	20.33	16.60	14.40
Ccl3	36.25	19.30	5.46	6.99
Gp38	32.72	16.98	6.97	5.08

Figure 5-2: Identification of potential gene promoters for brain metastases-specific expression of therapeutic molecules in infiltrating myeloid cells. (A) After whole body irradiation the bone marrow of C57BL6/J mice was reconstituted by HSCs from UbC-GFP mice. **(B)** Reconstitution was confirmed by flow cytometry analysis of mice leukocytes. **(C)** Gating strategy for the isolation of GFP^+CD11b^+ cells from brain metastases by FACS. Purple gate shows GFP^+CD11b^+ population that was isolated from brain tumour samples by FACS. **(D)** A heatmap of top 150 probes differentially expressed between bone marrow-derived myeloid cells ($CD11b^+GFP^+$) isolated from brain metastases (Br) and myeloid cells isolated from the spleen (S) or bone marrow (BM) (magnification is shown in Appendix A). **(E)** Fold changes in gene expression levels for the top 20 genes that are highly up-regulated in brain tumour infiltrating myeloid cells compared with myeloid cells in spleens and bone marrow across both tumour models (grey= top candidates for further analysis).

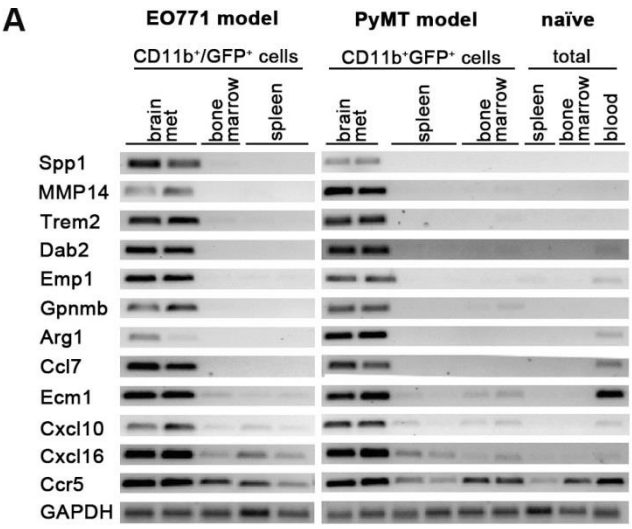
5.5 Validation of gene expression profiling in myeloid cells

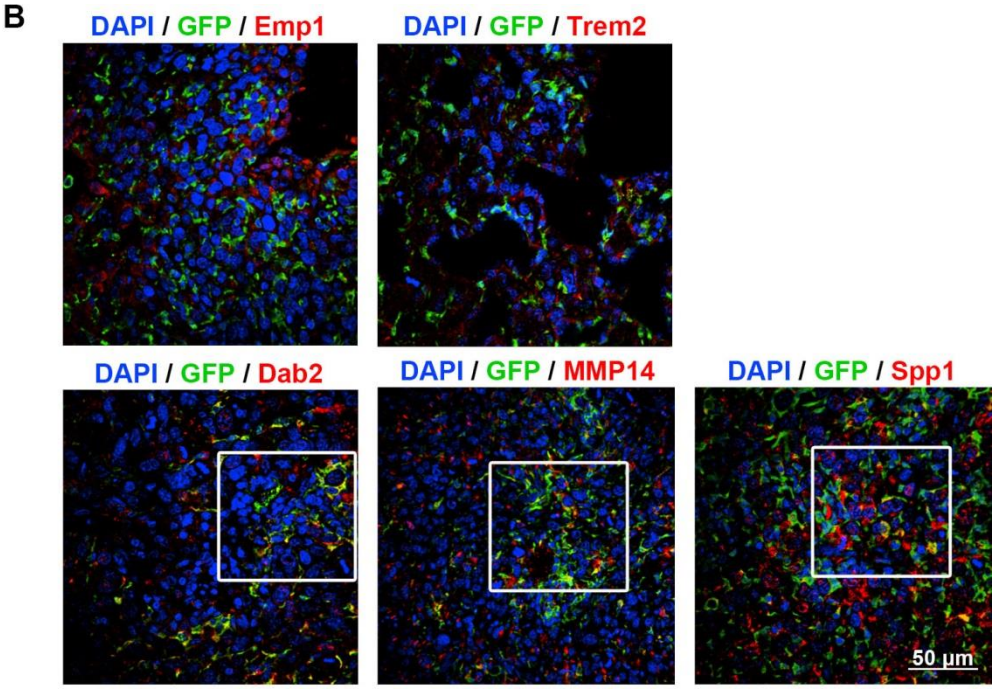
Gene expression analysis revealed that some genes are strongly up-regulated in $CD11b^+GFP^+$ cells in brain metastases compared with $CD11b^+$ cells in spleen and bm in both tumour models. Promoters of these genes could be used to control the specific expression of therapeutic molecules in a HSC-based cell therapy that could treat brain metastases. Based on different selection criteria 12 gene candidates were selected for further analysis. To validate the results of the gene expression analysis for these candidates a semi-quantitative PCR was performed. Expression levels were analysed in $CD11b^+$ cells isolated from brain metastases, bone marrow and the spleen, as well as in total spleen, total bone marrow and total blood cells. Some of the genes showed specific

expression in brain metastases-infiltrating CD11b$^+$ cells (e.g. osteopontin (Spp1), MMP14), while some of them also expressed in other tissues (e.g. Cxcl16, Ccr5; Figure 5-3A). The band intensities of the gel pictures were analysed with ImageJ and normalised to GAPDH band intensities (see Appendix B). Some genes were excluded from further analysis, since expression could be detected in other tissues (Gpnmb, Arg1, Ccl7, Cxcl16, Cxcl10, Ecm1). Spp1, MMP14, Trem2, Dab2 and Emp1 were genes that showed the highest specificity in brain metastases-infiltrating CD11b$^+$ cells by semi-quantitative PCR. Those five candidates were, therefore, chosen for further validation by immunofluorescence.

Brain metastases tissue isolated from the intracranial EO771 model in mice that have received GFP-transduced HSCs was analysed by IF for the expression of Spp1, MMP14, Trem2, Dab2 and Emp1 (Figure 5-3C). The GFP$^+$ brain metastases-infiltrating cells clearly expressed Dab2, MMP14 and Spp1, whereas the expression of Trem2 and Emp1 was restricted to cancer cells and there was no co-localisation with GFP. Based on these results, the expression of Dab2, MMP14 and Spp1 was further analysed in the murine spleen and bone marrow (Figure 5-3D). By contrast to the GFP$^+$ cells infiltrating brain metastases, the GFP$^+$ cells within the spleen and bone marrow exhibited no expression of Dab2, MMP14 or Spp1. Since the data indicated specific expression in the brain metastases-infiltrating HSC progeny, these three gene promoters were potential candidates that could be used to drive brain-metastases specific expression of therapeutic agents in a cell-based therapy.

Moreover, as expression of these three genes was shown to be specific in bone marrow-derived brain tumour-infiltrating cells we can postulate this expression is specific to bm-derived TAMs rather than brain-resident microglia. Therefore, the expression of these genes is assumed to be TAM-specific.


A
EO771 model
PyMT model
naïve
CD11b+/GFP+ cells
CD11b+GFP+ cells
total
brain met
bone marrow
spleen
brain met
spleen
bone marrow
spleen
bone marrow
blood
Spp1
MMP14
Trem2
Dab2
Emp1
Gpnmb
Arg1
Ccl7
Ecm1
Cxcl10
Cxcl16
Ccr5
GAPDH


B
DAPI / GFP / Emp1
DAPI / GFP / Trem2
DAPI / GFP / Dab2
DAPI / GFP / MMP14
DAPI / GFP / Spp1
50 µm

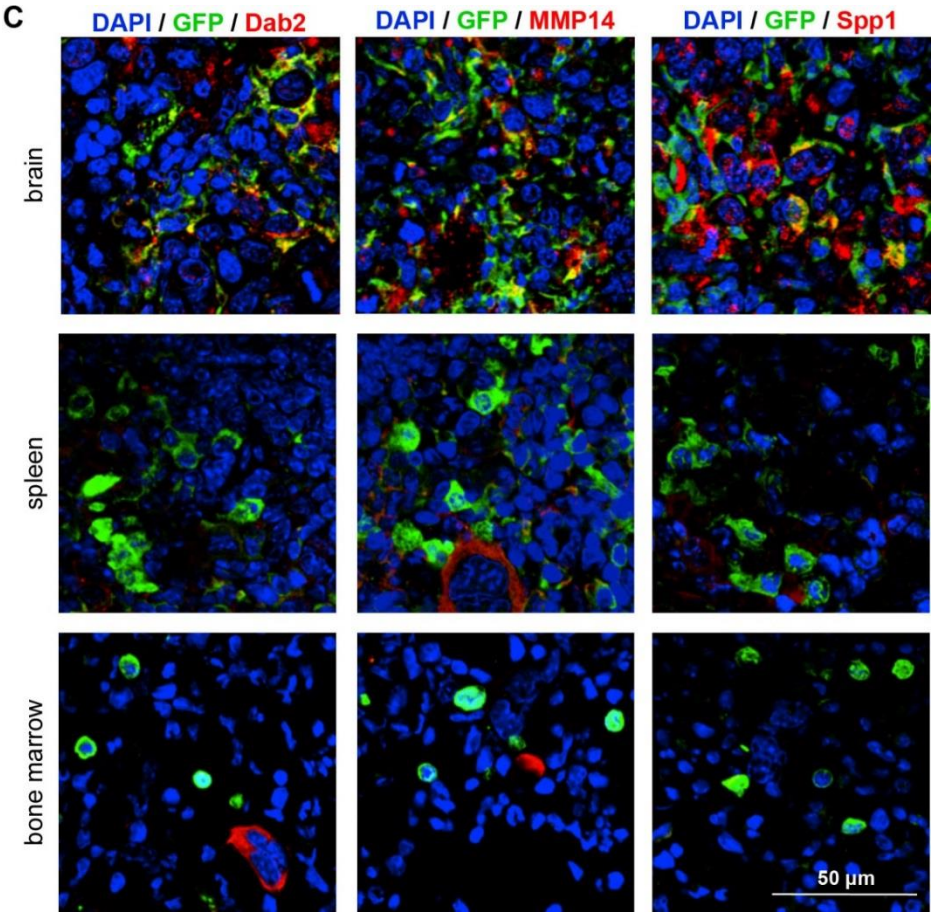
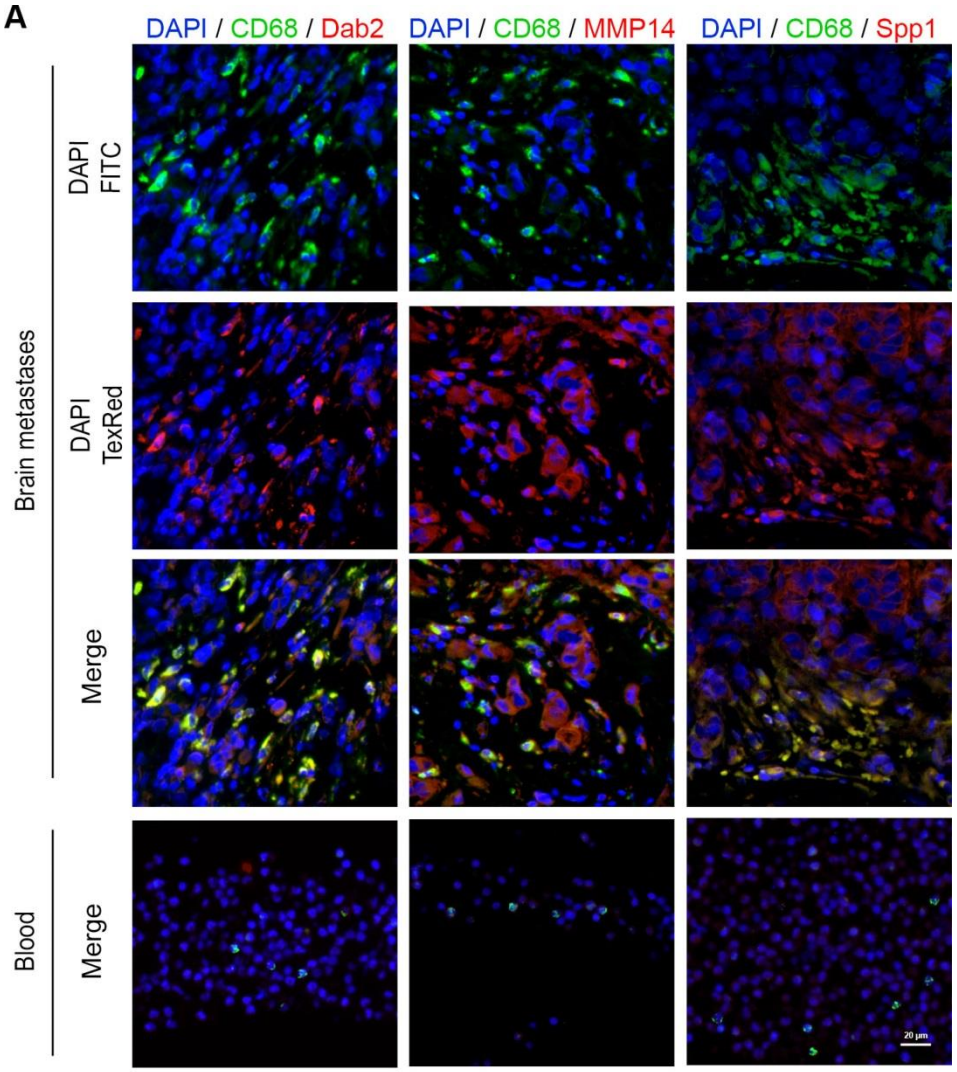
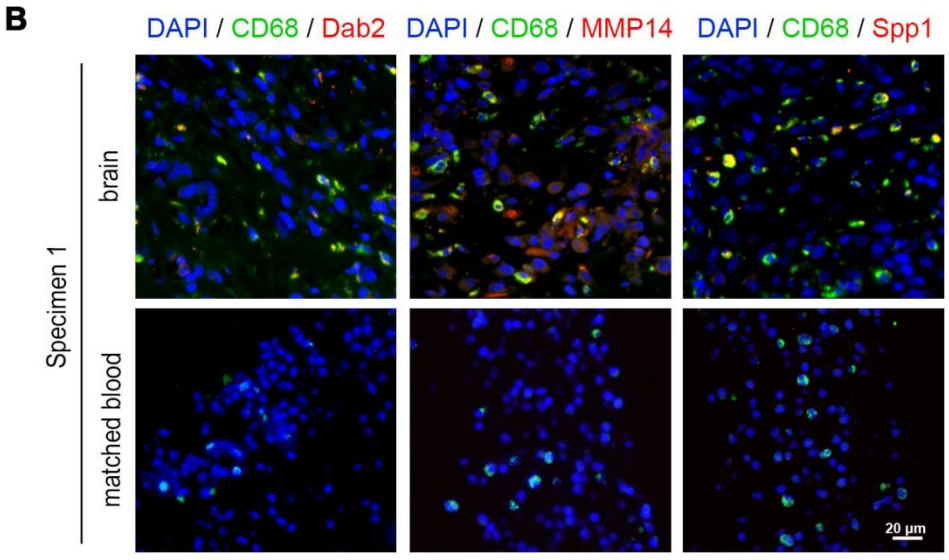


Figure 5-3: Validation of potential TAM-specific gene promoters in mice. (A) Semi-quantitative RT-PCR was used to validate the expression of the top 12 genes up-regulated in brain tumour TAMs (samples of GEX experiment and further blood, spleen and bm samples of naïve mice were used). **(B)** IF analysis of top five TAM-specific promoter candidates on protein level was performed on EO771 brain metastases tissue of mice that received GFP^+ HSCs. Co-staining for DAPI (blue), GFP (green) and Emp1, Trem2, Dab2, MMP14 or Spp1 (red) is shown. (Scale bar = 50 µm, original magnification 60x, representative images of two independent experimental replicates are shown). Larger magnifications of images stained for Dab2, MMP14 and Spp1 (white rectangles) are shown in (C), top row. **(C)** GFP^+ bone marrow-derived TAMs express Dab2, MMP14 and Spp1 whereas GFP^+ cells in the spleen and bone marrow display no expression of these three proteins. (Scale bar = 50 µm, original magnification 60x, representative images of two independent experimental replicates are shown).

To validate the translational potential of the cell-based therapy using the identified gene promoters, it was determined whether the three genes were specifically activated in macrophages infiltrating human brain metastases. To this end, four different specimens of patient-derived human brain metastases tissue and donor-matched blood were co-stained for CD68 and for Dab2, MMP14 or Spp1 (Figure 5-4). The $CD68^+$ microglia/macrophages in the four brain metastases specimens expressed all three proteins, while no co-expression could be detected in the donor-matched blood. This demonstrated a potential for the application of Dab2, MMP14 and Spp1 gene promoters in cell-based therapy in patients.


A
DAPI / CD68 / Dab2
DAPI / CD68 / MMP14
DAPI / CD68 / Spp1
Brain metastases
DAPI FITC
DAPI TexRed
Merge
Blood
Merge
20 µm


B
DAPI / CD68 / Dab2
DAPI / CD68 / MMP14
DAPI / CD68 / Spp1
Specimen 1
brain
matched blood
20 µm

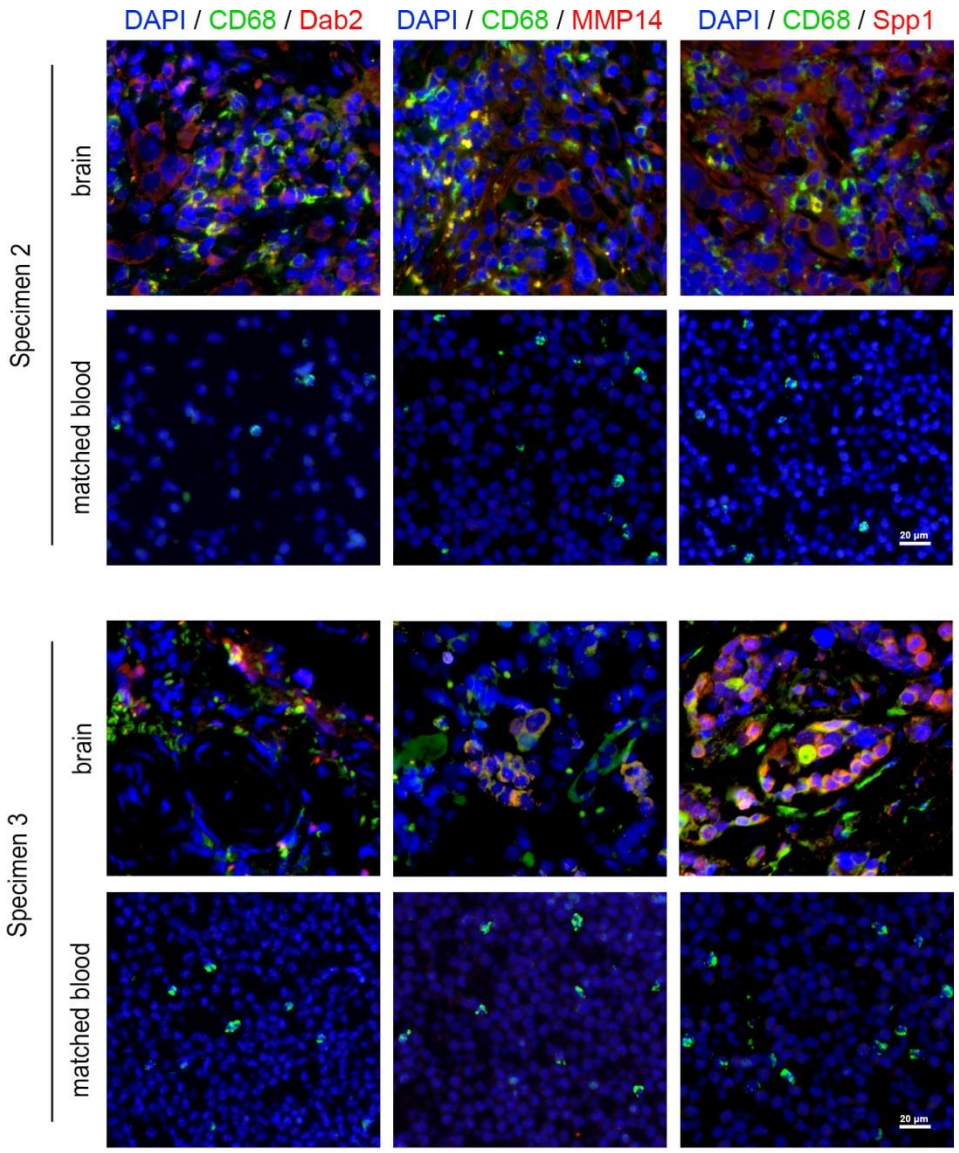


Figure 5-4: Validation of potential TAM-specific gene promoters in patient brain metastases samples. (A) IF co-staining for the macrophage marker CD68 (green) and Dab2, MMP14 or Spp1 (red) was performed on human brain metastases tissue and donor-matched blood. TAMs display expression of these three proteins, whereas blood macrophages do not. IF analysis of three further brain metastases specimens with donor-matched blood samples are shown in **(B)**. (Scale bar = 20 µm, original magnification 20x).

5.6 Analysis of TAM-specific promoter-reporter constructs

5.6.1 Analysis of promoter regions sequences

This study identified three genes specifically up-regulated in murine and human brain metastases-infiltrating HSC progeny whose promoters could be used to drive brain-metastases specific expression of therapeutic agents. To further functionally validate the

identified gene promoter candidates, lentiviral promoter-reporter constructs (lentiviral backbone: pEZX-LvPM02) of Dab2, MMP14 and Spp1 were purchased from GeneCopoeia.

To test the suitability of these promoter constructs, an analysis of the promoter regions of the purchased constructs was performed. Promoters are highly structured regulatory DNA elements that direct complex expression patterns in many different cell types specifically to each gene. Many genes contain binding sites for transcription factors located just 5′ from the core promoter, although additional regulatory DNA elements, such as enhancers, silencers and insulators, can be scattered over a distance of 100 kb. To identify consensus binding sequences for transcription factors and core promoter features in the promoter clones, a sequence analysis based on published sequences of murine promoter regions of the three genes Dab2, MMP14 and Spp1 was performed (Figure 5-5). The GeneCopoeia Dab2-promoter clone comprises a 1338 bp sequence of the mouse Dab2 promoter region from -1155 to +183 (Figure 5-5A). This sequence entails several putative cis-regulatory elements, which are binding sites for transcriptional activators, such as nuclear factor-I (NF-I), specificity protein 1 (Sp1), the octamer transcription factor 1 (OCT1), GATA-1 and GATA-3 as well as for the activator protein 1 (AP-1). Furthermore, this sequence contains a binding site for the basic helix-loop-helix protein (HEB), a binding site of POU-homeodomain factor and a glucocorticoid-responsive element (GR). The 5'-flanking sequence of the murine MMP14 gene is covered in the GeneCopoeia MMP14 promoter clone from position -1437 to -194 (Figure 5-5B). MMP14 has multiple transcription start sites (TSSs) and 3 of these are covered in the promoter clone sequence. Further, the promoter sequence entails several binding sites for transcription factors, such as Sp1, early growth response 1 (Egr-1), AP4, Nkx-2 as well as a CArG-box, which is a core binding site for the serum response factor (SRF) and other DNA-binding proteins. The Spp1-promoter clone comprises the 5'-flanking sequence from position -1044 to +239 of the murine Spp1 gene (Figure 5-5C). Many transcription factors can bind to the Spp1 clone, e.g. cAMP-response element (CRE), AP-1, 3, 4 and 5, PEA2 and 3. A positive regulatory 24 bp Vitamin D response element (Noda et al., 1990) located 761 bp upstream of the TSS is also included in this promoter sequence. Ras-activated enhancer (RAE) have been reported to increase Spp1 expression (Guo et al., 1995) following binding to the RAE sequence, which is also included in the reporter clone sequence.

All three promoter-reporter clones used in this study contain promoter elements crucial for the initiation of transcription, such as transcription start site and binding sites for putative cis-regulatory transcription factors.

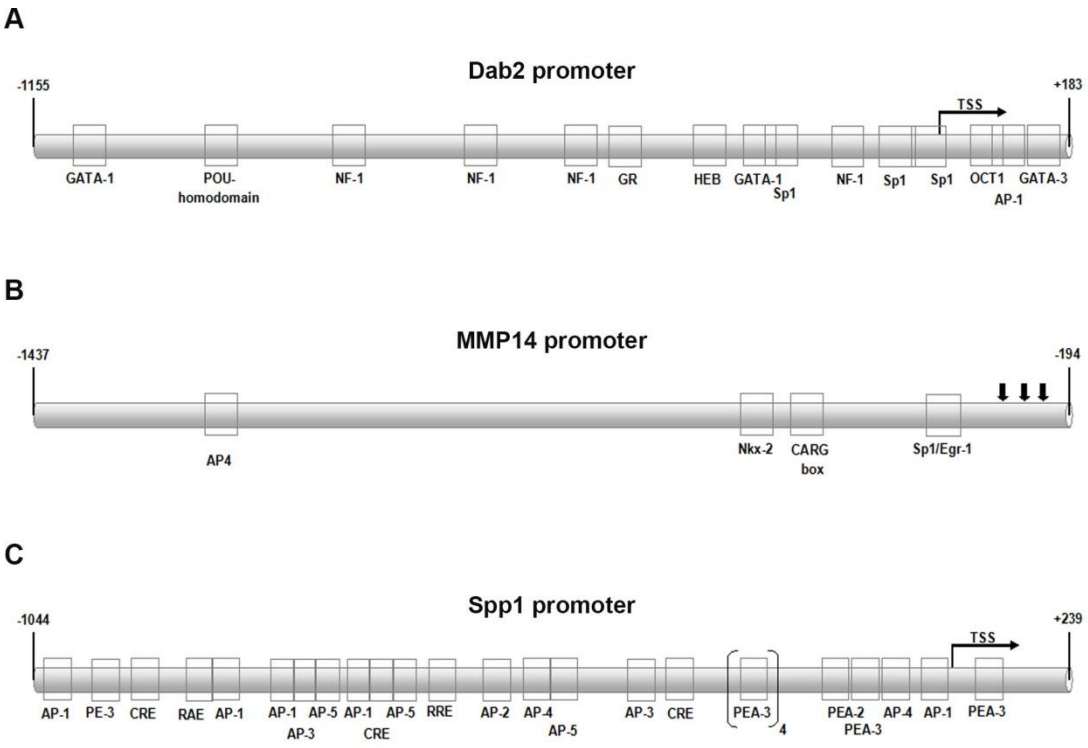


Figure 5-5: Illustration of the murine promoters and putative cis-regulatory elements of the promoter-reporter constructs of GeneCopoeia. (A) The Dab2-promoter clone entails the 5'-flanking sequence from -1155 to +183 of the murine Dab2 gene. Annotations are based on Cho and Park (2000) and Sheng et al. (2001). **(B)** The 5'-flanking sequence from -1437 to -194 of the murine MMP14 gene is covered in the GeneCopoeia MMP14-promoter clone. Locations of transcription factor binding sites and multiple transcription start sites (indicated by arrows) are based on Haas et al. (1999) **(C)** The Spp1-promoter clone comprises the 5'-flanking sequence from -1044 to +239 of the murine Spp1 gene. Annotations are based on Guo et al. (1995). The location of the Dab2 and Spp1 gene transcription start sites (TSS) is labelled by an arrow together with TSS. The putative binding sites for transcription activators are boxed.

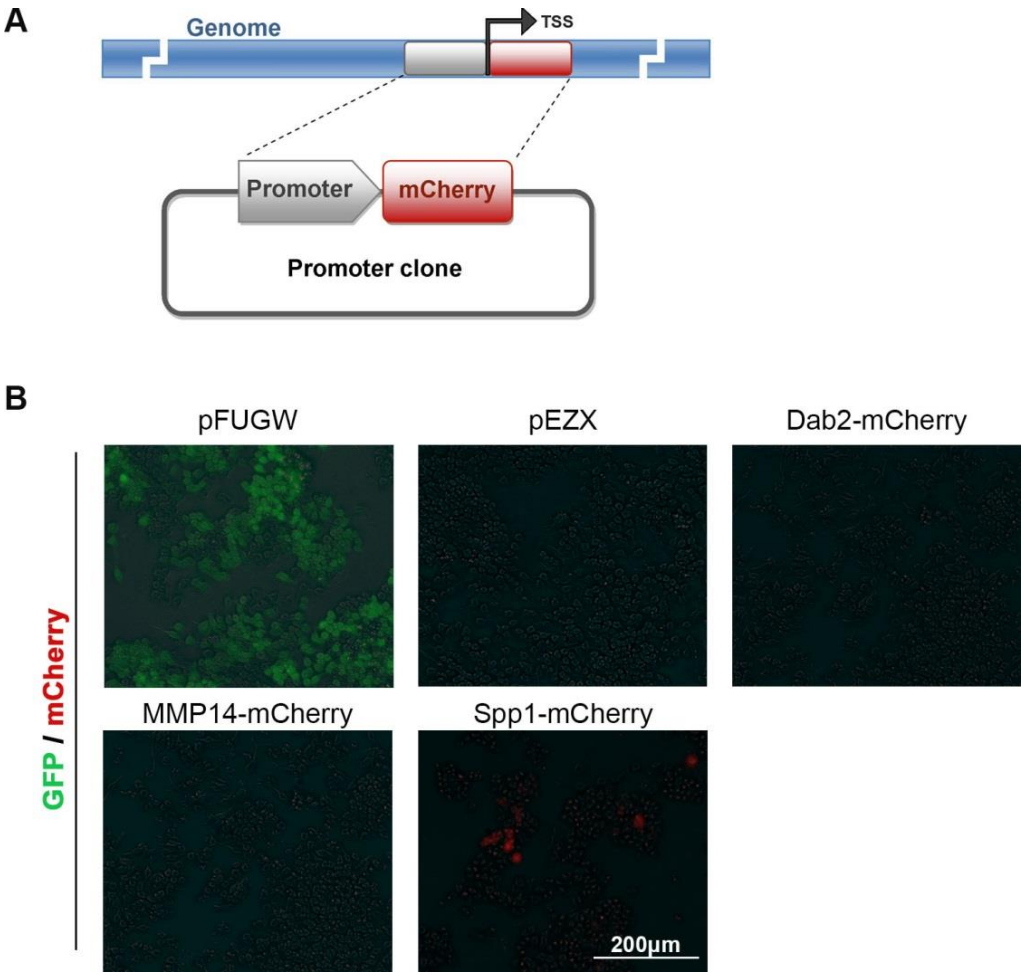
5.6.2 *In vitro* analysis of TAM-specific promoter-reporter constructs

As shown in Figure 5-6A, following transduction of cells with lentiviral virus particles of the three purchased promoter-reporter constructs, the DNA sequence, which is integrated into the genome contains the specific murine promoter controlling the expression of the reporter gene mCherry. To test these promoter clones *in vitro*, lentiviral particles were produced and the virus titre was determined using the reverse transcriptase assay.

HEK293 cells transduced with the promoter clones (Dab2-mCherry, MMP14-mCherry and Spp1-mCherry) were examined by fluorescence microscopy (data not shown). This analysis indicated that murine promoters were not activated in this human cell line of

non-haematopoietic origin. To test whether the murine promoters were active in a murine cell line of haematopoietic origin, the murine monocyte-macrophage cell line RAW 264.7 was transduced with the promoter clones (Figure 5-6B). To establish a transduction protocol for this cell line, RAW 264.7 cells were also transduced with pFUGW at an MOI30. The efficiency of this transduction was approximately 90% (Figure 5-6B) and this MOI was used for the transduction of this cell line with the promoter-reporter constructs and the empty vector control (pEZX). Analysis of the transduced RAW 264.7 cells by fluorescence microscopy revealed that only the Spp1 promoter was activated in RAW 264.7 cells (approximately 10%).

To further test the *in vitro* functionality of the remaining promoter-reporter constructs, induction tests were performed (Figure 5-6C). Despite the known induction of the murine Dab2 promoter by retinoic acid (Cho and Park, 2000, Smith et al., 2001), the Dab2-mCherry transduced RAW 264.7 cells did not show any expression of mCherry following addition of retinoic acid. Furthermore, published data has shown that the MMP14 promoter activity can be enhanced following exposure to EGF and TNF-α (Zhang et al., 2009, Han et al., 2001), but addition of the latter to transduced RAW 264.7 cells did not activate our MMP14 promoter. The enhanced Spp1 promoter activation following TNF-α stimulation, as published by Nakama et al. (1998), was also not detectable for the Spp1-mCherry transduced cells in our system. Lastly, the *in vitro* functionality of the promoter clones was tested by activation of macrophages with LPS (Meng and Lowell, 1997), which also failed to activate the promoters.


A
Genome
TSS
Promoter
mCherry
Promoter clone
B
pFUGW
pEZX
Dab2-mCherry
GFP / mCherry
MMP14-mCherry
Spp1-mCherry
200μm

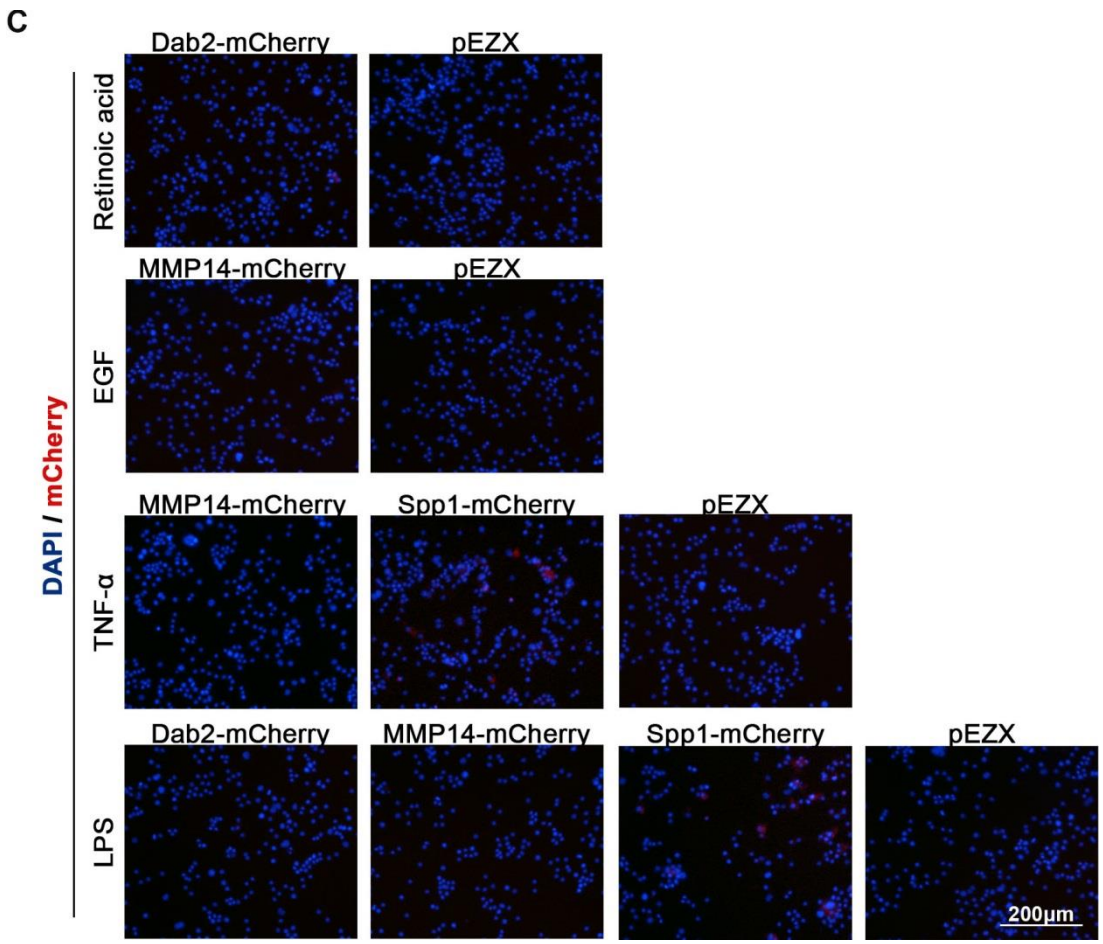


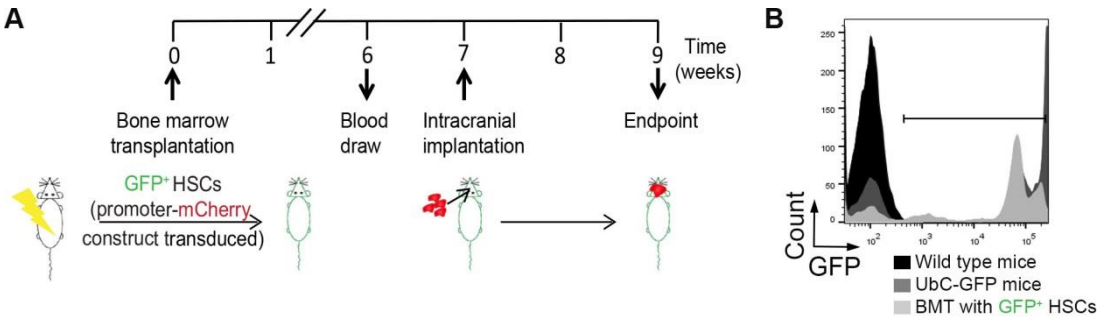
Figure 5-6: *In vitro* analysis of TAM-specific promoter-reporter constructs. (A) Illustration of promoter-reporter clones purchased from GeneCopoeia. **(B)** Fluorescence microscopy analysis of RAW 264.7 cells transduced with promoter-reporter constructs (Dab2-, MMP14- and Spp1-mCherry), pFUGW and pEZX (original magnification 20x, scalebar: 200 µm). **(C)** RAW 264.7 cells transduced with promoter-reporter constructs were treated with different transcriptional activators for three days (2 µM Retinoic acid, 100 ng/mL EGF, 10 µg/mL TNF-α and 0.25 µg/mL LPS) and subsequently analysed by fluorescence microscopy (original magnification 20x, scalebar: 200 µm, representative images of three independent experiments are shown).

5.6.3 *In vivo* analysis of TAM-specific promoter-reporter constructs

In vitro analysis did not demonstrate functionality of the three promoter clones. This could be due to missing factors of the tumour microenvironment. Therefore, *in vivo* functionality of the three promoter clones was tested next. The outline of this experiment is shown in Figure 5-7A. Firstly, a bone marrow transplantation was performed for eight female C57BL6/J mice. To this end, Scal$^+$ HSCs were isolated via MACS from the bone marrow of transgenic UbC-GFP mice. The isolated cells were transduced with a concentrated virus of promoter clones (Dab2-mCherry, MMP14-mCherry, Spp1-mCherry and pEZX) at MOI 30 overnight. One day after the whole body irradiation was performed, the eight mice received an iv injection of 1x10^6 promoter clone-transduced GFP$^+$ HSCs (Dab2:

n=2, MPP14: n=2, Spp1: n=2, pEZX: n=2). Six weeks after the BMT, blood was drawn from the tail vein of the mice and analysed by flow cytometry (Figure 5-7B). This was performed to determine if the bone marrow was successfully reconstituted by the transplanted HSCs. About 65-86% of all leukocytes in chimeric mice were GFP^+, compared with wild type (0.3%) and UbC-GFP mice (89%). The mCherry expression in leukocytes of these chimeric mice was also analysed by flow cytometry (Figure 5-7C). The mice that received HSCs transduced with Dab2 or MMP14 promoter constructs did not show any mCherry expression in their leukocytes, whereas a small $mCherry^+$ cell population (2.4% and 4.3%) could be detected in the leukocytes of mice that received Spp1-mCherry-transduced HSCs.

Subsequently, $1x10^5$ EO771 cancer cells were implanted into the mice brains. After the injected cancer cells formed large tumours, the tumour and a variety of different organs (brain tumour, bone marrow, lungs and spleen) were dissociated and analysed by flow cytometry (Figure 5-7D). To analyse the mCherry expression in the progeny of engrafted HSCs, the samples were stained for the marker of haematopoietic cells, CD45. The HSC progeny infiltrating brain tumours were negative for mCherry expression for all promoter clones, suggesting that tumour microenvironment did not activate Dab2, MMP14 or Spp1 promoters. The small percentage of $mCherry^+$ cells, detectable in the blood of mice that received Spp1-mCherry transduced HSCs, were not visible in the HSC progeny infiltrating brain metastases. Furthermore, no mCherry expression could be detected in the $CD45^+$ cells within other analysed tissues.



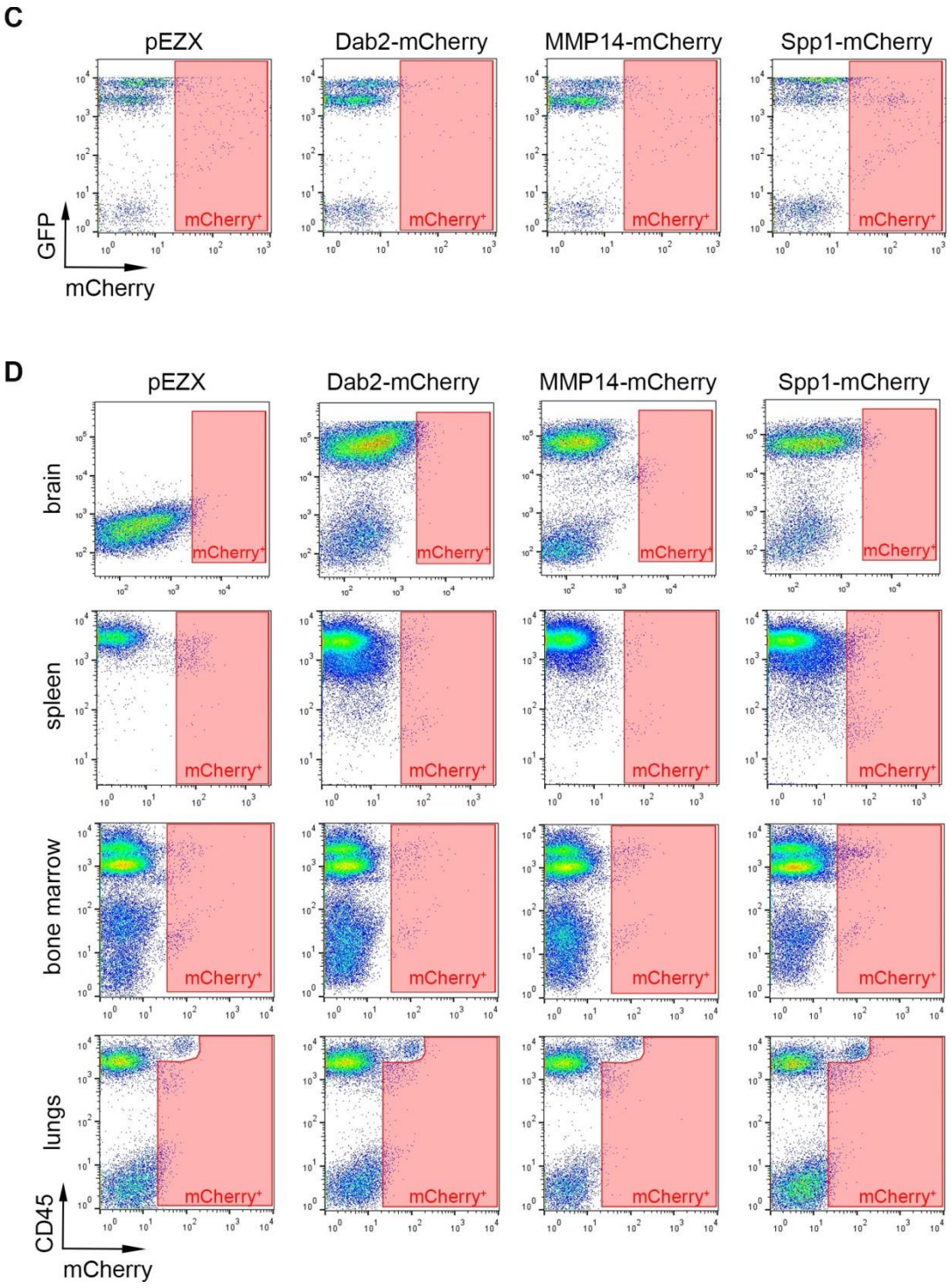


Figure 5-7: *In vivo* analysis of TAM-specific promoter-reporter constructs. (A) Experimental outline: intracranial implantation of EO771 cells was performed seven weeks after mice underwent BMT with GFP$^+$HSCs transduced with promoter-mCherry constructs. **(B)** The blood of experimental mice was analysed by flow cytometry to check bm reconstitution six weeks after BMT. **(C)** Further flow cytometry analysis of blood was done to check for mCherry expression in leukocytes (n=2 for each group (Dab2, MPP14, Spp1 and pEZX)). **(D)** Flow cytometry of HSC progeny in different tissues was performed (brain tumour, lungs, liver, spleen and bone marrow) to analyse its mCherry expression (n=2 mice for each group (Dab2, MPP14, Spp1 and pEZX)).

5.6.4 Troubleshooting the transduction efficiency

No mCherry expression could be detected in RAW 264.7 cells that were transduced with lentiviral reporter promoter constructs except in the Spp1-promoter clone. Furthermore, the incubation of the transduced cells with promoter-specific inducers did not lead to induction of mCherry expression. Additionally, no mCherry expression was detectable in brain tumours of mice that had received HSCs transduced with promoter-reporter constructs. Therefore, an investigation into why the expression of mCherry was not induced *in vitro* or *in vivo* was performed.

It was determined how well the promoter-reporter constructs were integrated into the genome of cells following transduction with the lentivirus, as low transduction efficiency has been a problem in previous experiments (4.3.4 and 3.5). To this end, HEK293 cells were transduced with lentiviral stocks of the promoter constructs at MOI 30. For this experiment the same virus stocks were used as in chapter 5.6.2 and 5.6.3. Three days later, genomic DNA was isolated from $1x10^6$ cells per sample. The number of viral integration sites per cell was determined by qPCR using primers that bind specifically to proviral insertion junctions (Table 5-1).

Table 5-1: Number of provirus integrations sites per cell for each sample determined by perfoming the Lenti-X provirus quantitation kit.

Cell line	Virus	Provirus copy number/cell
HEK293	-	0.29
HEK293	pEZX	22.60
HEK293	pEZX Dab2-mCherry	6.13
HEK293	pEZX MMP14-mCherry	3.64
HEK293	pEZX Spp1-mCherry	86.15
EO771	pFUW Fluc	17.10

Non-transduced HEK293 cells served as a negative control for this experiment. 0.29 virus integration sites per cell could be detected for this sample. The established cell line EO771/FLuc, routinely used by the work group and known for consistent and strong expression of the transgene FLuc, was used for this experiment as a positive control. Approximately 17 virus copies per cell were determined for this sample. The provirus copy numbers for the promoter clone samples ranged from 3.64 to 86.15. This result indicated that promoter construct-transduced cells should have a sufficient amount of transgene sequences integrated into the genome to perform reporter expression analysis.

5.7 Discussion

The major drawback of the HSC-based therapy approach presented in this study is the low intracranial specificity of the delivery due to the naturally occurring accumulation of the HSC progeny in organs such as the spleen, lungs, liver, blood and bone marrow. An infiltration of GFP^+ cells in blood (45%), bone marrow (25%), spleen (20%), lungs (20%) and liver (10%) could be detected in mice 6 weeks after a bone marrow engraftment with pFUGW transduced HSCs. Thus, once genetically engineered HSCs expressing a therapeutic agent are used in this cell-based therapy approach, the agent would accumulate in brain metastases as well as other organs, potentially leading to systemic toxicities.

Gene promoters specifically up-regulated in TAMs infiltrating brain metastases could be used to drive the brain metastases-specific expression of therapeutic molecules within TAMs and thereby limit the delivery of therapeutic agents exclusively to brain lesions. Moreover, these cell-specific promoters may provide a level of expression better adapted to cellular metabolism, facilitating a longer-term expression of the therapeutic molecule. Restricting gene expression to a specific cell population through the use of a cellular-specific promoter is also expected to reduce a potential immunogenicity of the cell-based therapy.

To identify such cell-specific gene promoters for the cell-based gene therapy, a genome-wide gene expression analysis of bm-derived $CD11b^+$ cells isolated from brain metastases, bone marrow and the spleen of chimeric mice with GFP^+ bone marrow was performed. Genes were identified that were specifically up-regulated in the brain metastases-infiltrating myeloid cells and subsequently their specific expression patterns were validated on murine and human tissue samples.

Expression patterns of candidate genes that have been identified to be specifically up-regulated in brain metastases-infiltrating TAMs were analysed and confirmed on murine and human tissue samples. Thus, three genes were identified whose promoters could be used to drive brain-metastases specific expression of therapeutic agents. To test the functionality of the corresponding promoter constructs, lentiviral promoter-reporter clones of Dab2, MMP14 and Spp1 were purchased from GeneCopoeia. Subsequently, mCherry reporter expression analysis failed to detect the expression of mCherry *in vivo* in murine brain metastases after the engraftment of promoter clone-transduced HSCs, as well as in transduced murine RAW 264.7 cells following stimulation with known promoter activators. A mCherry expression was only detected in Spp1-reporter clone transduced RAW 264.7 cells. Further, whereas a small $mCherry^+$ leukocyte population

could be detected in the blood of mice that have received Spp1-mCherry-transduced HSCs (2.4% and 4.3%), mCherry expression was undetectable in the brain tumours of these mice. The fact that mCherry expression was only detectable in Spp1 clone-transduced cells and not in Dab2 or MMP14 clone-transduced cells could be explained by the number of viral integration sites per transduced cell. This number, determined by using the Lenti-X provirus quantitation kit on transduced cells with the same MOI was more than ten times higher for Spp1 (86.15) than for Dab2 (6.13) and MMP14 (3.64). An integration of the promoter-reporter sequence into the genome of three to six times per cell should be sufficient to perform reporter expression analysis. However, the results of the integration site quantification analysis also revealed that although all samples were transduced at the same MOI under the same experimental conditions (same backbone and similar promoter lengths), the transduction efficiency varied between the samples. Therefore, it is evident that an alternative method should be considered to determine the initial virus titre.

Promoter sequence analysis revealed that all promoter clones contained crucial elements to initiate transcription of the reporter gene, although no expression was detectable. The Dab2 promoter clone contains the 5'-flanking sequence of the murine Dab2 gene from position -1155 to +183 to regulate the expression of the reporter gene mCherry. This sequence contains several positive-regulatory binding sites for transcription factors including GATA binding sites. The binding of GATA factors is known to induce Dab2 expression, and GATA-dependent Dab2 expression can be induced by the presence of retinoic acid (Smith et al., 2001). Capo-Chichi et al. (2010) identified and tested four GATA binding sites located upstream of the ATG site of the mouse Dab2 gene (−1904, −1926, −3943, −3894 bp). The two most potent of these GATA-binding sites −1904 and −1926 for GATA complex formation are not included in the promoter sequence from GeneCopoeia. Further, Rosenbauer et al. (2002) used for his study a Dab2 promoter-reporter clone, which contained a 2 kb insert of the 5'-genomic region of the murine Dab2 gene that also included the two most potent GATA-binding sites. With this study a novel role of Dab2 as an inducer of cell adhesion and spreading of macrophages was identified, suggesting a crucial role for these included GATA-binding sites (−1904 and −1926) in the Dab2 promoter activation. Therefore, the promoter sequence of the Dab2 clone should be reviewed and most probably extended to include the GATA-binding sites at position −1904 and −1926.

According to Haas et al. (1999), the murine MMP14 promoter clone (length -1437 to -194) lacks one of the 4 TSS, as well as binding sites for transcription factors such as NF-kB, c-myc, PEA-3,c-ETS1. Another study on cis-acting promoter elements of the murine

MMP14 gene by Cha et al. (2000) has shown that the strongest induction of reporter expression could be achieved with a promoter length from position -3000 to -99. Zhang and Brodt used a murine MMP14-reporter construct, which included the promoter sequences from the nucleotide position -1297 to –97 for their study in 2003. This promoter sequence included the binding sequence for type 1 insulin growth factor (IGF-1) leading to an increased reporter activity after IGF-1 treatment. With this study the influence of IGF-1 on MMP14 synthesis in the context of tumour invasion was demonstrated. Additionally, Ray et al. (2004) identified a binding region for positive-regulatory cis-acting elements mediating the presence of oxidised low-density lipoproteins within the position -213 and -1 of the 5'-flanking region of the murine MMP14 gene. All these studies used MMP14-reporter constructs that were different in promoter length but the sequence of the fourth TSS was included in all of them. Therefore, to optimise MMP14 promoter analysis for the cell-based therapy, editing of the purchased promoter clone to include this sequence should be considered.

The promoter of the Spp1 clone contains various binding sites for positive regulatory transcription factors and cells transduced with this promoter clone do express mCherry. However, this low expression could not be enhanced by known transcription inducers and is not stable *in vivo*. Therefore, to improve the Spp1 promoter performance, the promoter sequence of the purchased Spp1 clone should be altered. According to Guo et al. (1995), the GeneCopoeia Spp1 promoter clone (-1044 to +239) does not entail binding sites for nuclear factor ets-like (NF-E), CRE and PEA3, which are located further upstream of the TSS of Spp1. However, even shorter Spp1 promoter lengths have been successfully used for promoter studies. Zhao et al. (2010) used the murine promoter sequence from position -882 to +79 for their study on osteopontin expression in macrophages, whereas Guo et al. (2001) used the sequence -777 to +79 of the murine Spp1 gene for promoter analysis for their study on nitric oxide synthesis in macrophages. Interestingly, the promoter sequences in both the above mentioned studies ended at position +79, while the promoter sequence of the GeneCopoeia clone ends at position +239. The possibility of a negative-regulatory binding site present in the sequence between +79 and +239 should be explored by performing promoter studies with a shortened Spp1-reporter clone.

The use of cell type or tissue-specific promoters for gene therapy has already been explored by other research groups (Hatzoglou et al., 1990, Maxwell et al., 1991, Vile and Hart, 1993, Chen et al., 1995, Arbuthnot et al., 1996, Lee et al., 1996, Abdallah et al., 1996). Even though cellular-specific promoter driven suicide gene therapy showed promising results, leaky expression of the tissue specific promoters in non-targeted cells

has been reported (Gommans et al., 2006). Therefore, alternative strategies may be essential in addition to the cell-type specific promoter regulation of the gene therapy. The use of microRNA (miRNA) regulation, adjacent to the use of the cell type-specific promoter, could provide another layer of control by which transgene expression can be restricted to brain metastases TAMs in contrast to other M2-like macrophages at other sites. MiRNAs are small, non-coding RNAs, that regulate various cellular functions and are known to mediate RNA interference (RNAi) to posttranscriptionally regulate gene expression is well known (Wang and Lee, 2009). Binding of endogenous miRNAs to complementary miRNA-targeting elements (miRTs) promotes translational inactivation and/or RNA degradation. Each cell contains a diverse population of miRNAs, and the abundance of individual miRNA varies greatly between different cell lineages and different tissues. Introducing miRTs that are recognised by a cell type-specific miRNA, into the 3'UTR (untranslated region) of the transgene of the gene therapy vector could be an effective way to knock down the expression of a therapeutic gene in undesired cell types. Recently, an approach allowed the transgene expression only in targeted glioblastoma cancer cells without any expression in normal astrocytic cells of the same lineage. To this end, Wu et al. (2009) engineered the glial fibrillary acidic protein (GFAP) promoter driven tissue specific gene expression vector along with the target sequences for several deregulated miRNAs that were introduced into the 3'UTR of the transgene. Another tissue-specific, miRNA-regulated dual control suicide gene therapy vector was engineered by Danda et al. (2013) to selectively express the suicide gene in EpCAM overexpressing cells. Normal retinal glial cells overexpress let-7 miRNAs and let-7b expression was found to be down regulated in retinoblastoma tumours. Therefore, let-7b miRNA target sequences were cloned into the 3'UTR of HSV TK suicide gene driven by EpCAM promoter.

The use of cell-specific promoters in gene therapy ensures therapeutic effects in desired cells or tissues, and limits side effects caused by gene expression in non-target cells. However, cellular promoters in gene therapy have demonstrated relatively weak transcriptional activity compared with viral promoters (Nicklin et al., 2001, Liu et al., 2008). One strategy of enhancing the expression of transgenes is to combine the cell-specific promoter with a viral transcriptional regulatory element. Several studies added a CMV enhancer region 5' to a cell-specific promoter to increase its transcriptional activity (Robinson et al., 1995, Yew et al., 1997, Sawicki et al., 1998, Yew et al., 2001, Liu et al., 2004). Another strategy to enhance the activity of cell-specific promoters without the loss of the cell type specificity is transcriptional amplification strategy (TAS). This strategy uses the cell-specific promoter to drive simultaneous expression of the

desired transgene and a strong artificial transcriptional activator to potentiate transcription by binding to the specific binding sites introduced into the promoter. This strategy has been implemented successfully in gene therapies to enhance transgene expression from weak cell-specific promoters (Nettelbeck et al., 1998, Iyer et al., 2001, Liu et al., 2006). However, lentiviral vectors have a modest packaging capacity of approximately 8 kb (Davidson and Breakefield, 2003), which limits this dual promoter system when longer promoters (e.g. > 2 kb) have to be used.

Further investigations are needed to find suitable promoter candidates to drive the tissue-specific expression of therapeutic molecules in the cell-based therapy to treat brain metastases, including promoter studies with altered promoter clones as discussed. Furthermore, additional regulatory DNA elements, such as enhancers, silencers and insulators that can be scattered over a distance of 100 kb for each gene should be investigated and their possible incorporation into the expression vectors considered.

6. Discussion and future work

Treatment options for brain metastases are strongly limited by the poor access of drugs into the brain (Lockman et al., 2010). This study demonstrated the development of an HSC-based therapy that has the potential to overcome these limitations. To this end, HSCs and their progeny were exploited as cellular delivery vehicles for the delivery of genetically encoded therapeutic molecules into brain metastases.

First, brain metastases-infiltrating cell populations in preclinical brain metastases models were investigated. The homing of microglia/macrophages to brain metastases has already been demonstrated by various studies (He et al., 2006, Davoust et al., 2008, Lorger and Felding-Habermann, 2010). To elucidate the contribution of the two myeloid cell populations in brain metastases individually, a flow cytometry analysis of murine brain metastases tissue was performed. This allowed us to distinguish between macrophages and microglia. The analysis revealed that brain metastases were predominantly infiltrated by myeloid cells and the majority of these cells expressed high levels of CD45 and CD11b, characteristic of macrophages (Davoust et al., 2008, Sedgwick et al., 1991). Analysis of patient-derived brain metastases tissue also indicated strong presence of macrophages. Notably, although immunofluorescence analysis does not allow a clear distinction between macrophages and microglia, the majority of $CD68^+$ cells in human brain metastases tissue showed elongated spindle-shape morphology, which has been shown to be characteristic of macrophages (Yamasaki et al., 2014).

Phenotypic analysis of brain metastases in chimeric mice with GFP-tagged bm demonstrated that the dominant infiltrating myeloid cell population originates from the bone marrow. Moreover, this demonstrated the efficient delivery of GFP to brain lesions by the myeloid progeny of HSCs that have been genetically engineered to express GFP. Therefore this experiment served as a proof-of-principle for the HSC-based cell therapy targeting brain metastases.

Following the demonstration of GFP delivery to brain lesion by the HSC progeny, it was further investigated whether an enzyme prodrug approach had a therapeutic effect on experimental brain metastases. However, constitutive expression of the bacterial enzyme by the HSC progeny, in combination with prodrug administration, showed no detectable therapeutic effect in brain metastases-bearing mice. This may have been due to the possible immunogenic potential of constitutively expressed enzyme. Therefore, the constitutively active UbC promoter was replaced by a Dox-inducible promoter. The functionality of the inducible enzyme prodrug system was successfully demonstrated *in vitro* and *in vivo*. However, this approach still needs to be tested in the *in vivo* HSC-based therapy setting.

In addition to homing to brain metastases, the progeny of HSCs have also been shown to infiltrate other organs. In the context of the cell therapy this could lead to the accumulation of therapeutic molecules at those sites, resulting in possible side effects of the therapy. To address this issue, three promoters with high specificity and activity in brain metastases-infiltrating TAMs were identified. These promoters could be used to restrict the delivery of genetically encoded therapeutic agents to brain metastases. So far, only one of the tested promoter-reporter constructs showed activity *in vitro* and *in vivo*. Therefore, promoter sequences need to be further optimised to identify optimal promoter constructs for the HSC-based therapy. Notably, all three promoter candidates also showed specific activity in macrophages infiltrating human brain metastases as opposed to the donor-matched blood.

To conclude, this study demonstrated the feasibility of experimental HSC-based therapy to treat brain metastases and a potential for its translation into the clinic. The latter was substantiated by strong infiltration of macrophages into human brain metastases, as well as by the translatability of identified brain metastases-specific promoter candidates into human setting.

6.1 Future work

In various ways, the results of this thesis could potentially be used for further development of the cell-based therapy.

The developed inducible CD/5-FC approach still needs to be tested in the *in vivo* HSC-based therapy setting. Once therapeutic efficacy in brain metastases is demonstrated, this therapeutic approach could be investigated in a cell-based therapy to treat brain metastases using human HSCs in immunocompromised mice, such as NOD/SCID/IL2rγKO.

Further, as brain tumour specificity of this cell therapy could be demonstrated, a large spectrum of other therapeutic molecules can be delivered to target brain metastases. Examples for possible therapeutic options include cytokines or chemokines for immunomodulation, monoclonal antibodies, or receptor-targeting secreted peptides. The therapeutic effect of these on experimental brain metastases has to be analysed first.

Throughout this study low lentiviral transduction efficiency was a problem. Higher reconstitution rates, as demonstrated by others (Pawliuk et al., 2001, Tabatabai et al., 2010), could not be achieved. It has already been discussed that the Lenti-X provirus

quantitation kit represents a better method to determine virus titre. Moreover, to achieve higher transduction efficiency different steps of the lentiviral transduction protocol can be modified, which has been discussed in chapter 3.7.

Additionally, the tumorigenic potential of transduced HSCs should also be investigated. To this end, the insertion sites of the transgene should be analysed towards their proximity to proto-oncogenes or tumour suppressors.

Sequences of promoters that have been demonstrated to be specifically active in brain metastases-infiltrating TAMs have to be optimised to ensure activity as well as cell type-specific expression *in vitro* and *in vivo*. These sequences can then be used to establish the cell-based therapy with increased specificity. Thereby, these promoters could drive tissue-specific expression of therapeutic molecules in the brain tumour-infiltrating progeny of the administered HSCs.

The TAM-specific promoters could also be used for other investigations. For example, the contribution of macrophages and microglia to brain tumours could be investigated individually. There are two studies where depletion of myeloid cells had opposing effects on experimental glioma growth (Galarneau et al., 2007, Markovic et al., 2009). Both studies used the same transgenic mouse model. In those studies the thymidine kinase gene was under the control of the myeloid-specific CD11b promoter, which allows depletion of myeloid cells following the administration of the prodrug ganciclovir (Gowing et al., 2006). The discrepancy in results could be due to different routes of ganciclovir administration, which could have caused the depletion of different cell types. However, these studies could be improved by the use of promoters that are specific to the individual myeloid cell populations, macrophages and microglia. In this thesis, brain metastases TAM-specific promoters (Dab2, MMP14 and Spp1) have been identified. If it can be demonstrated that these promoters are not active in microglia, they could be used for this purpose.

Moreover, with this study TAMs have been identified to be the largest bm-derived brain metastases-infiltrating population and their M2 phenotype (anti-inflammatory, pro-angiogenic and tumour-promoting properties) was assessed. Different strategies in regard to these findings can be applied in a cell-based therapy to treat brain metastases. For example, macrophages rather than HSCs could be directly used as cellular delivery vehicles. Thereby, the cell-based therapy would be more time efficient, as the process of a BMT is not required. To this end, *in vivo* studies of tumour-tropism, half-life, best site of administration and amount of injected and genetically engineered macrophages has to be investigated.

Further, the HSC-based cell therapy could also be combined with a strategy to re-polarise M2-like TAMs into tumour-suppressive M1 TAMs or to therapeutically delete the M2-like TAMs. Various options for each of these strategies have been discussed in chapter 3.7. These therapeutic strategies have to be tested *in vitro* first before their therapeutic efficacy in combination with the HSC-based cell therapy in preclinical brain metastasis models can be established. To avoid any potential effects on other leukocyte populations caused by TAM-targeted HSC-based therapies, target specificity would also need to be addressed. Target specificity could be achieved with brain metastases TAM-specific promoters.

In conclusion, there are many further avenues of investigation that can be pursued from this study. This work, if continued, may lead to a clinical therapy to treat brain metastases.

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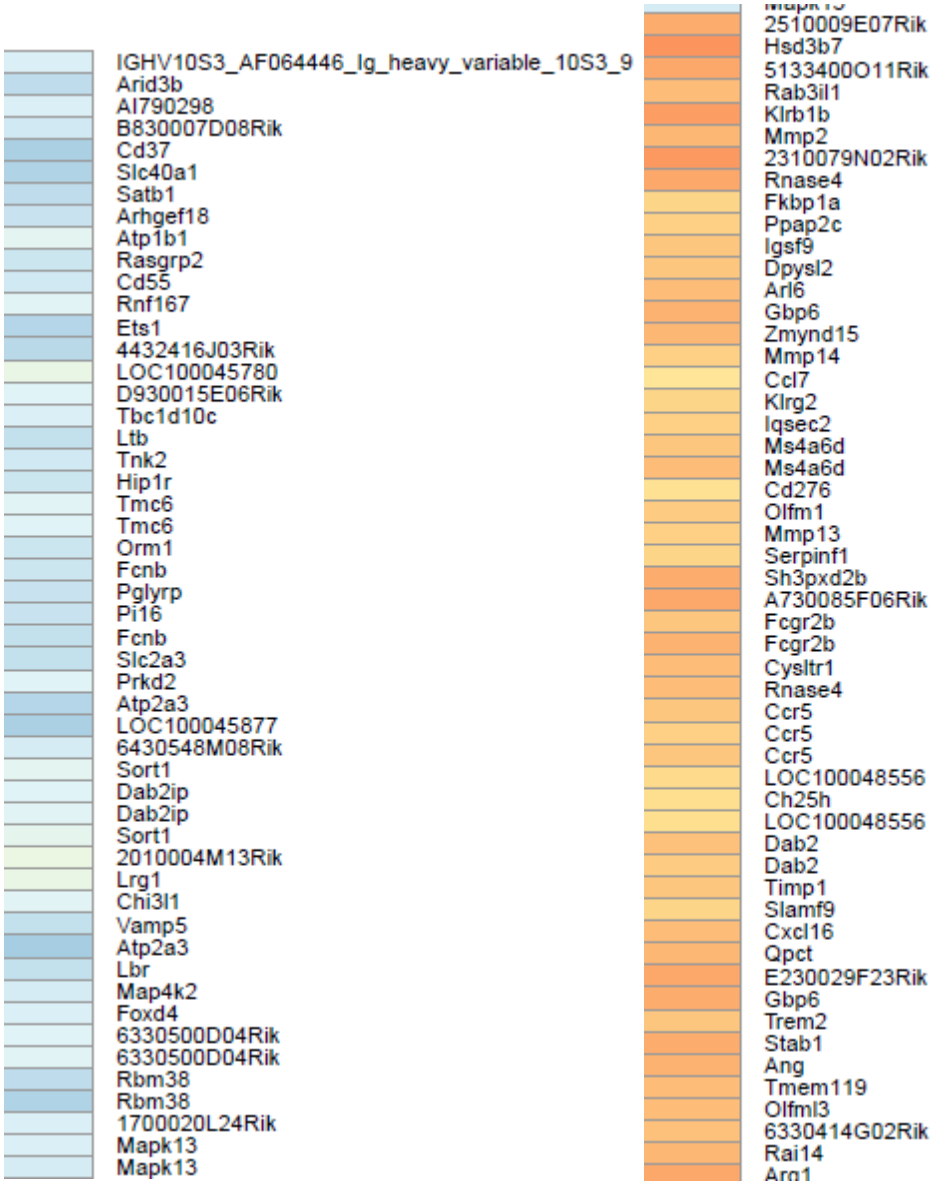
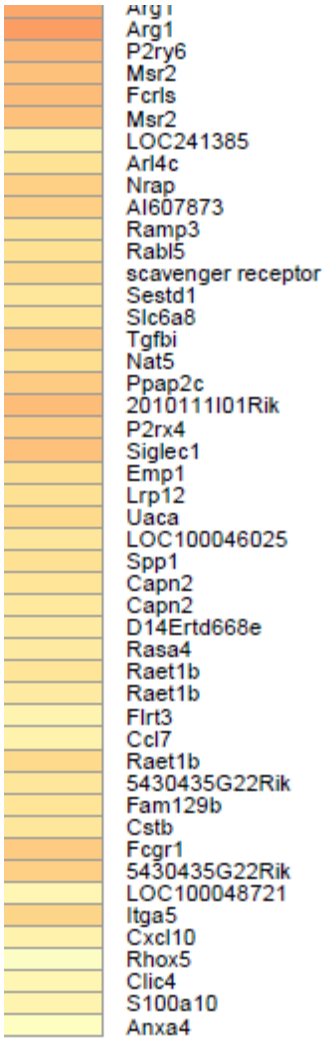
Appendix A

The top 150 differentially expressed probes between myeloid cells of different tissues

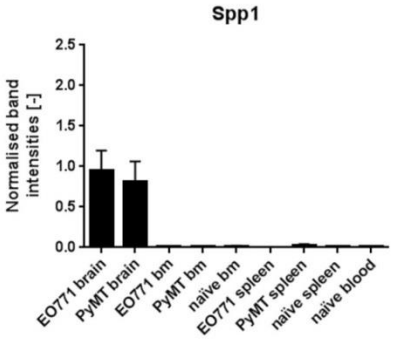




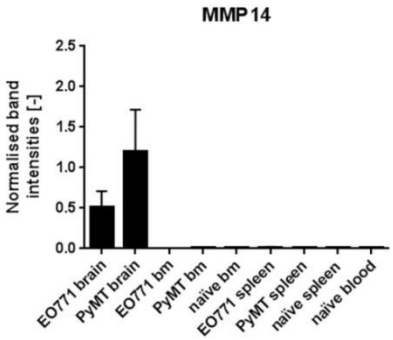
Supplemental Figure S 1: List of the top 150 probes differentially expressed between bone marrow-derived myeloid cells isolated from brain metastases and myeloid cells isolated from the spleen or bone marrow (magnification of heatmap of Figure 5-2D)**.**

Appendix B

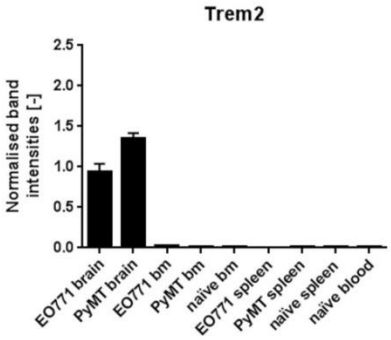
Quantification of semiquantitative RT-PCR



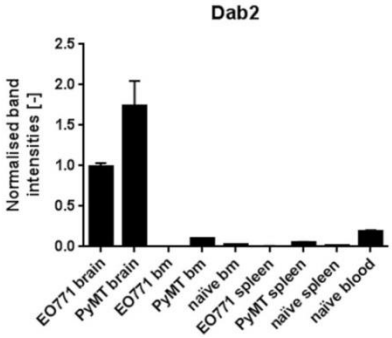
vs.	EO771 brain	PyMT brain
EO771 brain	-	ns
PyMT brain	ns	-
EO771 bm	***	***
PyMT bm	***	***
Naïve bm	***	***
EO771 spleen	***	***
PyMT spleen	***	***
Naïve spleen	***	***
Naïve blood	***	***



vs.	EO771 brain	PyMT brain
EO771 brain	-	*
PyMT brain	*	-
EO771 bm	ns	***
PyMT bm	ns	***
Naïve bm	ns	***
EO771 spleen	ns	***
PyMT spleen	ns	***
Naïve spleen	ns	***
Naïve blood	ns	***

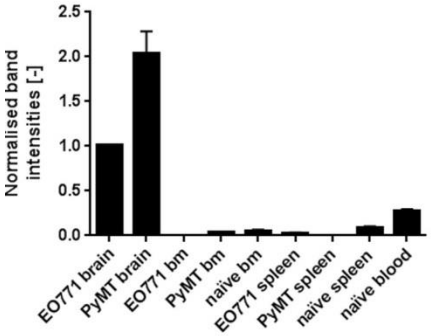


vs.	EO771 brain	PyMT brain
EO771 brain	-	****
PyMT brain	****	-
EO771 bm	****	****
PyMT bm	****	****
Naïve bm	****	****
EO771 spleen	****	****
PyMT spleen	****	****
Naïve spleen	****	****
Naïve blood	****	****



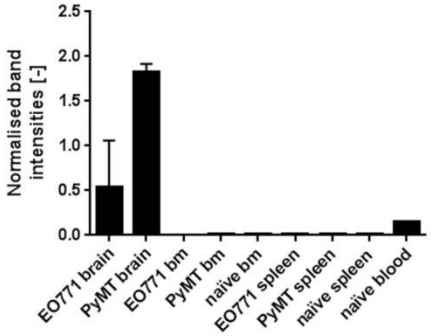
vs.	EO771 brain	PyMT brain
EO771 brain	-	***
PyMT brain	***	-
EO771 bm	****	****
PyMT bm	***	****
Naïve bm	****	****
EO771 spleen	****	****
PyMT spleen	****	****
Naïve spleen	****	****
Naïve blood	***	****

Emp1



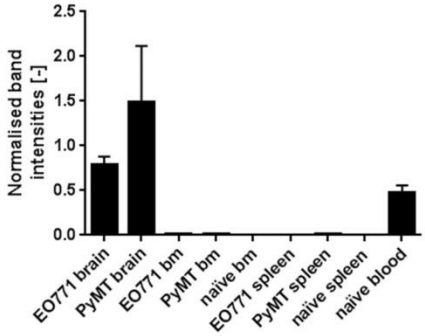
vs.	EO771 brain	PyMT brain
EO771 brain	-	****
PyMT brain	****	-
EO771 bm	****	****
PyMT bm	****	****
Naïve bm	****	****
EO771 spleen	****	****
PyMT spleen	****	****
Naïve spleen	****	****
Naïve blood	****	****

Arg1



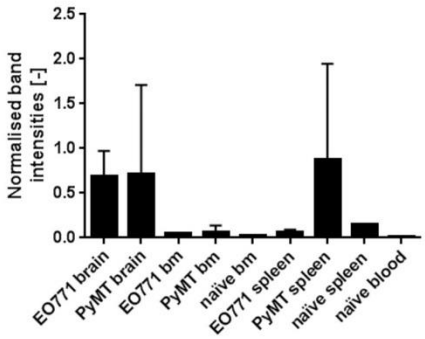
vs.	EO771 brain	PyMT brain
EO771 brain	-	***
PyMT brain	***	-
EO771 bm	ns	****
PyMT bm	ns	****
Naïve bm	ns	****
EO771 spleen	ns	****
PyMT spleen	ns	****
Naïve spleen	ns	****
Naïve blood	ns	****

Ccl7

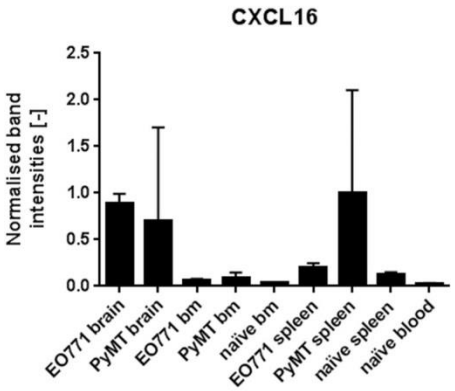


vs.	EO771 brain	PyMT brain
EO771 brain	-	*
PyMT brain	*	-
EO771 bm	*	***
PyMT bm	*	***
Naïve bm	*	***
EO771 spleen	*	***
PyMT spleen	*	***
Naïve spleen	*	***
Naïve blood	ns	**

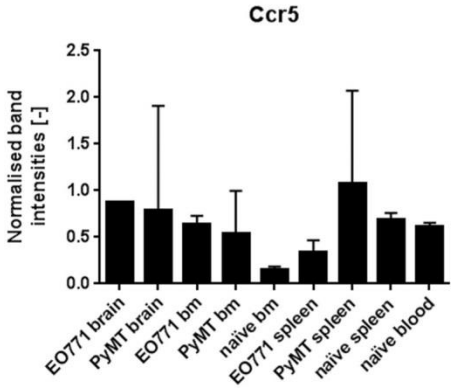
CXCL10



vs.	EO771 brain	PyMT brain
EO771 brain	-	ns
PyMT brain	ns	-
EO771 bm	ns	ns
PyMT bm	ns	ns
Naïve bm	ns	ns
EO771 spleen	ns	ns
PyMT spleen	ns	ns
Naïve spleen	ns	ns
Naïve blood	ns	ns



vs.	EO771 brain	PyMT brain
EO771 brain	-	ns
PyMT brain	ns	-
EO771 bm	ns	ns
PyMT bm	ns	ns
Naïve bm	ns	ns
EO771 spleen	ns	ns
PyMT spleen	ns	ns
Naïve spleen	ns	ns
Naïve blood	ns	ns



vs.	EO771 brain	PyMT brain
EO771 brain	-	ns
PyMT brain	ns	-
EO771 bm	ns	ns
PyMT bm	ns	ns
Naïve bm	ns	ns
EO771 spleen	ns	ns
PyMT spleen	ns	ns
Naïve spleen	ns	ns
Naïve blood	ns	ns

Supplemental Figure S 2: Quantification of semi-quantitative RT-PCR (Figure 5.3A). The band intensities of the gel pictures were analysed with ImageJ and normalised to GAPDH band intensities. Statistical significance determined with one-way ANOVA using the Dunnett's multiple comparisons method, with alpha=5% (ns: not significant, *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001).

Appendix C

Sequences of plasmids

pFUW-sCD

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Cytosine deaminase tPA

pFUW-sCDHA

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HA-tag

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Cytosine deaminase

tPA

HA-tag