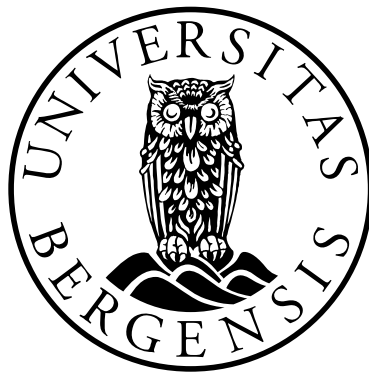# Melanoma brain metastasis

Animal models, Detection and Therapy interventions

Inderjit Kaur Daphu

Dissertation for the degree of philosophiae doctor (PhD)

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II

Scientific Environment

The presented work was carried out at the Department of Biomedicine, University of Bergen, during the period January 2009 - March 2013, under the supervision of Professor Frits Thorsen and Professor Rolf Bjerkvig. The PhD fellowship was granted by the Faculty of Medicine and Dentistry, at the University of Bergen. The experimental work was supported by the Norwegian Cancer Society, the Norwegian Research Council and Helse-Vest, Haukeland University Hospital, Bergen.

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My special thanks goes to Anja Torsvik, Inger-Anne Netland, Amra Grundic-Feta, Linda Sleire and Agnete S. T. Engelsen for their lovely and supportive nature. Thanks for the scientific discussions and breaks together. It was fun, sharing office with you, Michael Marie and thanks for all Photoshop discussions.

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My research and this doctorate degree is dedicated to my Mom and Dad, who have been a great source of inspiration whole my life and will be further in the future. Dad your support and moral has never decreased a bit, even though you supply the good wishes and encouragement through phone calls now. A big thanks goes to my brother and sisters for their encouragement and supportive talks on long phone calls. I would also like to thank my mother-in-law for her dinners; it was a great help through the busy times.

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are a wonderful son, your hugs and love filled my energy tanks whenever I come home. At last but not the least thanks goes to my child in my tummy who has written whole thesis with me, struggling long nights and hard busy days.

Bergen, March 2013

Inderjit Daphu

Table of Contents

VIII

List of Publications

This thesis is based upon the following papers, which are referred in the text by their Roman numerals.

Paper I **Daphu I**, Sundstrøm T, Horn S, Huszthy PC, Niclou SP, Sakariassen PO, Immervoll H, Miletic H, Bjerkvig R, Thorsen F.

In vivo animal models for studying brain metastasis: value and limitations.

Clin Exp Metastasis. 2013 Jun;30(5):695-710

Paper II Wang J*, **Daphu I***, Pedersen PH, Miletic H, Hovland R, Mørk S, Bjerkvig R, Tiron C, McCormack E, Micklem D, Lorens JB, Immervoll H, Thorsen F.

A novel brain metastases model developed in immunodeficient rats closely mimics the growth of metastatic brain tumours in patients.

Neuropathol Appl Neurobiol. 2011 Feb;37(2):189-205

* These authors have contributed equally to this publication.

Paper III Sundstrøm T, **Daphu I**, Wendelbo I, Hodneland E, Lundervold A, Immervoll H, Skaftnesmo KO, Jendelova P, Babic M, Sykova E, Johansen ML, Bjerkvig R, Thorsen F.

Automated tracking of nanoparticle-labeled melanoma cells improves the predictive power of a brain metastasis model.

Cancer Res. *2013 Apr 15;73(8):2445-56*

Paper IV **Daphu I**, Horn S, Stieber D, Varughese J, Skaftnesmo KO, Bjerkvig R, Thorsen F.

Inhibition of human melanoma brain metastatic growth *in vitro* by targeting the MAPK and PI3K signaling pathways.

Manuscript

Abbreviations

aCGH	Array-comparative genomic hybridization
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
BLI	Bioluminescence imaging
BRAF	Proto-oncogene B-Raf
c-Kit	Human proto-oncogene c-kit
Co	Cobalt
CNS	Central Nervous system
CT	Computed tomography
CTLA	Cytotoxic T-lymphocyte antigen
DNA	Deoxyribonucleic acid
DTIC	Dacarbazine
ECM	Extra cellular matrix
FDA	Food and Drug Administration
GEMMs	Genetically engineered mouse models
GFP	Green fluorescent protein
GFAP	Glial fibrillary acidic protein
GSK	Glaxo Smith Kline
Gy	Gray (unit of radiation)
HIF-1	Hypoxia inducible factor 1
ICA	Intracarotid
ICD	Intracardial
IV	Intravenous
KPS	Karnofsky performance status
MAPK	Mitogen-activated protein kinase
MGd	Motexafin gadolinium
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging

mTOR	Mammalian target of rapamycin
NK cells	Natural killer cells
pAKT	Phosphorylated serine/threonine-specific protein kinase
PDGF	Platelet derived growth factor
PET	Positron emission tomography
PI3K	Phosphoinositide 3-kinase
PLX4032	Vemurafenib, BRAFV600E inhibitor
pMAPK	Phosphorylated mitogen-activated protein kinase
p-mTOR	Phosphorylated mammalian target of rapamycin
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
ROS	Free oxygen radicals
RPA	Recursive-partitioning analysis
RSR13	Efeproxiral
RTOG	Radiation therapy oncology group
SPIONs	Superparamagnetic iron oxide nanoparticles
SRS	Stereotactic radiosurgery
T cell	Lymphocytes maturing in thymus gland
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WBRT	Whole brain radiation therapy

Abstract

This thesis is comprised of three research papers and one review paper. In paper I, we recapitulated previous literature on animal models used to study brain metastasis. Many of the currently available models have shed knowledge on underlying metastatic mechanisms, although none of them fully reflect the human brain metastatic disease. In the research work of this thesis, we thus developed new animal models that could amend new information on the human brain metastatic disease.

In paper II, we developed a rat model where we successfully implanted human brain metastases spheroids from patients into the rat brain. Tumors developing in the rat brain showed strong similarities to the corresponding patient brain tumors. Thus, this model may be used to study biological mechanisms and treatment responses.

Paper III describes a robust and reproducible model system where we can track prelabeled human melanoma brain metastatic cells in the brain of a mouse model by T2*weighted MRI. Automated quantification of tumor cells in the brain increases the probability to predict tumor burden in the animal brain after intracardial inoculation and eventually exclude the animals with inoculation failures. This model represents an asset for increasing the success rate of preclinical animal experimental design.

Paper IV represents a therapeutic *in vitro* study on melanoma brain metastasis cells, which harbor both the BRAFV600E mutation and a PTEN deletion. We successfully targeted the MAPK and PI3K signaling pathways by combining PLX4032 (BRAFV600E inhibitor) and Temsirolimus (a specific mTOR inhibitor) therapies. The combined therapy showed a significantly synergistic inhibitory effect on tumor cell growth as compared to monotherapies. Moreover, global gene analyses indicated that functions related to cell cycle, cell-death and -survival, cellular movement and DNA-replication, -recombination and -repair are also affected by the combined treatment. Thus this study makes a foundation for our upcoming, preclinical therapy studies in animals, which in turn may form a fundament for future clinical therapeutic studies.

1. Introduction

1.1 Cancer

1.1.1 General aspects

A tumor is characterized by an abnormal, uncontrolled, proliferation of cells. Benign tumors are mostly confined to the tissue of origin, where their progression is characterized by a local expansive growth and in many cases they can be completely removed, by surgery. Malignant tumors, on the other hand, have the capacity to invade and destroy adjacent tissues, which eventually will lead to metastasis to the organs distant from their site of origin. This process involves tumor dissemination via the vascular or lymphatic system[1]. More than 90% of cancer deaths are associated with metastasis that frequently renders surgical resection ineffective as a therapeutic option[2,3]. A tumor at the site of origin is referred to as a primary tumor whereas a metastatic lesion developing in a distant organ is referred to as a secondary or metastatic tumor.

1.1.2 The global cancer burden

Cancer represents a major global health problem. According to statistics, more than 12 million patients are diagnosed with cancer annually, and accounts for over 7 million deaths world-wide[4]. In the United States, malignant neoplasms are ranked second after heart diseases, as the leading causes of death in 2009[5]. Moreover, the National Cancer Institute in USA has calculated the cost of cancer care in the US in 2010 to exceeded $ 124 billion[6].

1.1.3 Pathophysiology

Cancer represents a complex, multifactorial disease involving changes in the genome[7], orchestrated by intrinsic factors of the host genome (genetic predisposition) or by environmental factors[8]. Genomic alterations involve changes in the DNA sequence (mutations), DNA copy number alterations, chromosomal rearrangements
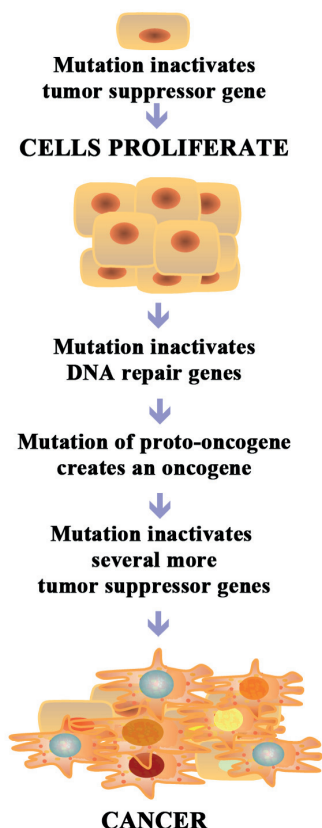
and epigenetic changes, which leads to tumor initiation and progression. The hallmarks of cancer, defined by Hanahan and Weinberg as: self-sufficiency in growth signals, insensitivity to anti-growth signals, ability for tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, and evasion of apoptosis[7]. These characteristics provide cancer cells with functional capabilities of progressive tumor growth[9]. In a sequel to the Cell publication in 2000, Hanahan and Weinberg recently also proposed emerging hallmarks as: deregulating cellular energy metabolism and avoidance of immune detection and destruction[3]. Genomic instability and tumor-promoting inflammatory reactions represents the major enabling characteristics that foster these hallmarks. Moreover, the tumor microenvironment, which is composed of various non-malignant cells, may contribute to tumor progression by secreting growth factors and extracellular matrix molecules[3,10].

1.1.4 Cancer initiation

That tumor initiation is a result of accumulated mutations in the cell's DNA was proposed by Carl O. Nordling in 1953[11], and later formulated by Alfred G. Knudson in 1971[12]. He proposed that multiple "hits" to DNA is necessary for malignant transformation. Based on his observations on inherited retinoblastoma, he proposed that the first insult to the DNA is inherited and that a second insult is necessary for malignant transformation to occur. Later it was acknowledged that malignant transformation depends both on an activation of proto-oncogenes (i.e genes that stimulate cell proliferation) and on a deactivation of tumor suppressor genes (i.e genes that suppress cell proliferation). This implies that a first "hit" in an oncogene not necessarily cause cancer, since normally functioning tumor suppressor genes will suppress tumor formation. However with damage also in a suppressor gene, uncontrolled cell proliferation may take place (Fig. 1)

Damage to the DNA may occur as a result of both exogenous as well as endogenous insults. Typical exogenous insults may be caused by various viruses, bacterias, radiation and carcinogenic chemicals, whereas endogenous insults may involve DNA damage induced by free oxygen radicals (ROS) and replication errors during DNA synthesis.

In the organism, cells cannot function if DNA damage corrupts the integrity and accessibility of essential information in the genome. Due to the hazard of genomic alterations, cells have developed defense mechanisms against DNA damage. Such defense mechanisms involve various DNA repair mechanisms, the induction of a state of dormancy known as senescence, induction of apoptotic programs and a destruction of damaged cells by the immune system[13,14].

Mutation inactivates tumor suppressor gene

CELLS PROLIFERATE

Mutation inactivates DNA repair genes

Mutation of proto-oncogene creates an oncogene

Mutation inactivates several more tumor suppressor genes

CANCER

Figure 1. Schematic presentation of cancer development as a result of a serial set of mutations in tumor suppressor genes and proto-oncogenes. Cancer development may start with an inactivation of tumor suppressor genes that may lead to increased cell proliferation. An inactivation of the DNA repair machinery together with mutations in proto-oncogenes may further lead to malignant progression.

1.1.5 The cellular evolution of cancer

Most tumors are derived from a single transformed cell and is thus of monoclonal origin. Yet, due to genetic instability, multiple tumor cell populations develop, leading to a divergent mass of heterogeneous tumor cells. In 1976, Nowell proposed that cancers evolve through multiple mutations, that through stochastic processes, lead to an accumulation and selection of genetic changes in tumor

subpopulations[15]. However, during the last ten years an old concept, based on the cancer stem cell theory of tumor progression, has received renewed attention[16-18]. This theory suggests that tumors are initiated and driven by cancer stem-like cells that through asymmetric cell divisions give rise to heterogeneous tumor cell populations. At present cancer stem-like cells have been identified in a variety of tumor types, including breast cancer[19], multiple myeloma[20], head and neck squamous cell carcinoma[21], pancreatic cancer[22], colon cancer[23], prostate cancer [24] and brain tumors[25,26]. Several genes and intracellular signaling pathways, required for normal stem cell function, have shown to be activated in cancer stem-like cells where they may have essential roles in tumor development and induction of therapy resistance[16,27].

When it comes to melanomas it is currently unclear to what extent cancer stem cells are involved in tumor initiation, since it has been shown in experimental systems that multiple tumor cell populations can have tumor initiating capacities[28].

1.2 The metastatic process

1.2.1 Definition and epidemiology

Cancer metastasis represents the terminal stages of cancer progression in a multi-step process[29]. Metastasis is the main cause of death for most cancer patients[30,31]. A distinct feature of metastatic disease is the ability for different primary cancers to colonize in either the same or different organs (Table 1). Metastasis is characterized by the spread of cancer cells from its primary location to other organs of the body. Localized spread to lymph nodes is not normally regarded as metastasis, however it is usually a sign of poor prognosis. Cancer cells break away from the primary tumor, penetrate into the blood stream or the lymphatic system, circulate, attach to and penetrate the vessels, and then grow in a new location in normal tissues elsewhere in the body.

A number of genes contributing to metastasis to different organs have been studied[32-35], but it is at present unclear if these are used by different tumor types to

metastasize to the same organs[36]. It is also observed clinically that some tumors have a restricted range of target organs than others. For instance, prostate cancer metastasis largely restricted to bone, while sarcomas commonly spread to the lungs (Table 1).

Tumor type	Main sites of metastasis
Breast	Bones, lungs, liver, brain
Lung adenocarcinoma	Brain, bones, adrenal gland, liver
Skin (melanoma)	Lungs, brain, skin, liver
Colorectal	Liver, lungs
Pancreatic	Liver, lungs
Prostate	Bones
Sarcoma	Lungs
Uveal melanoma	Liver

Table 1. Typical sites of metastasis for solid primary cancers. Adapted from Nguyen et al[36].

It is also important to acknowledge that the kinetics of tumor development differs between tumor types. Adenocarcinomas of the breast and the lung commonly metastasize to similar organ types such as bone, lung, liver and brain. However, breast cancer recurrences are commonly detected years or decades after initial treatment[37,38], while lung cancer metastasis occurs typically a few months after diagnosis[39,40]. This means that the ability for different cancer types to infiltrate distant organs is not accompanied by the same ability to colonize the organs. The mechanisms behind differences in the clinically observed metastatic latencies remain unknown[36].

1.2.2 The classical steps of tumor metastasis

Cancer metastasis is a multi-step process, where the cells from the primary cancer locally loose cellular adhesion and invade the basement membrane of the vasculature, followed by tumor cells breaking through the blood vessels or the lymphatic vessels (a process called intravasation). The cells then have to survive in the circulation, before they attach to the luminal, endothelial cells in the vasculature,

followed by a break-through into the target tissue (a process called extravasation), with subsequent colonization to form solid metastatic tumors[30,31,2,41].

Local invasion at the primary tumor site. The metastatic process starts when tumor cells invade into the extracellular matrix (ECM) of the primary cancer. This step may involve changes in the cell to cell and cell to ECM adherence involving changes in cell surface receptors and proteolytic degradation of ECM. These processes are followed by increased tumor cell motility.

Tumor cell adherence to ECM is mediated by integrins and cell-cell adhesions by cadherins. Integrins are heterodimers of 1 of 18 α and 1 of 8 β transmembrane proteins. Each heterodimer binds to specific proteins in the ECM and can transmit signals into or out of the cells[42]. Cadherins bind cells through homophilic protein-protein interactions of their extracellular domains and signal intracellularily to catenins and the actin cytoskeleton. Cadherin expression switches the invasion in tumor cells by turning E-cadherin, which promotes tumor cell-tumor cell adherence, to N-cadherin, which is normally expressed on mesenchymal cells and helps in tumor cell binding to the stroma during invasion[43].

Increased expression or an altered expression of proteases that degrade the ECM has also been associated with the invasive process. Matrix metalloproteinases, plasmin, urokinase plasminogen activator, cathepsins and heparanases are examples of proteases involved in the invasion process[44].

Chemokines also contribute to tumor invasion by inducing infiltration of macrophages and lymphocytes that release proteases, growth-, angiogenic- and immunosuppressive factors[45].

Tumor cell movement, also referred to as an ameboid movement process, is frequently a result of a loss of cellular polarity. This process is characterized by changes in cell shape directed by resistance in the surrounding normal tissue[44].

Systemic spread through the vasculature. Primary cancer cells that are able to intravasate, face a tough environment in the vasculature. Circulating immune cells can attack the tumor cells, and they are exposed to mechanical wearing forces due to

velocity. The cells also lack an attachment substratum[31]. Thus the metastatic process is highly inefficient, as the majority of cells undergo apoptosis[46]. It is estimated from animal experiments in our paper III, that less than 0.01% of tumor cells entering the bloodstream are able to survive and form metastatic tumors.

Extravasation. Tumor cells extravasate by inducing endothelial retraction, which lead to attachment of tumor cells to sub-endothelial ECM molecules. Many lines of evidence indicate that most tumor cells die within the circulatory system but some cells may overcome this hurdle[47]. Generally, tumor cells arrest in capillary beds or bind coagulation factors, including tissue factor, fibrinogen, fibrin and thrombin forming an embolus. The arrest of emboli in capillary beds may initiate the growth of tumor cells at the secondary site. In the lymphatic system, selectins may mediate a weak attachment of tumor cells to endothelium, where cadherins or immunoglobulin like cell adhesion molecules further strengthens the attachment of tumor cells to the endothelium[48,49].

Metastatic colonization. Successful colonization depends on interactions with the microenvironment or the "soil" of the distant tissue. In 1889, Stephan Paget published a novel observation in metastasis research[50]. His theory described tumor cells as the "seed" and the host environment as the "soil". Paget hypothesized that their interactions determine the metastatic outcome: "When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil". This observation predicted that the tissue microenvironment could facilitate the metastatic process contributing to tumor growth in specific organs.

There are many factors that can limit tumor progression within the tumor microenvironment. This include factors within the ECM, basement membranes, reactive oxygen species, limited availability of nutrients and oxygen, and interference by the immune system. For instance, the aggressiveness of primary tumors can be influenced by cellular responses to hypoxia. A low oxygen tension in cells can lead to the stabilization of the hypoxia inducible factor-1 (HIF-1) transcriptional complex that further activates genes responsible for angiogenesis, anaerobic metabolism, cell survival and invasion[51]. Tumors that shows abundant HIF-1 stabilization may show a

higher potential for metastasis[52]. Other microenvironmental factors may also drive the selective evolution of the primary tumors. This includes reactive nitrogen and oxygen species, which are generated by both infiltrating inflammatory cells and rapidly proliferating tumor cells. These factors induce genomic instability that leads to an expression of genes that facilitate the metastatic process[53].

1.2.3 Skin cancer

Skin cancer has become a significant health problem among the white Caucasian population, and the yearly incidence is increasing in the Western World[54,55]. The skin cancers are commonly divided into two groups: Melanomas, and the non-melanoma skin cancers (NMSC), consisting of basal cell carcinomas (BCC) and squamous cell carcinomas (SCC). In 2009, melanoma accounted for 44.9% of the total numbers of new skin cancers in Norway (males and females), but represents 88.6% of the total number of skin cancer related deaths ("Cancer in Norway 2009", the Cancer Registry of Norway) (Fig. 2).

Of all solid primary cancers, cutaneous malignant melanoma has one of the highest risks for developing brain metastases[56]. More than 40% of melanoma patients with advanced disease are treated for brain metastatic disease.

Figure 2. Yearly incidence of skin cancers (melanomas and non-melanomas) in Norway 2000-2009, and registered skin cancer deaths in Norway 2009. The data is obtained from "Cancer in Norway 2009", the Cancer Registry of Norway.
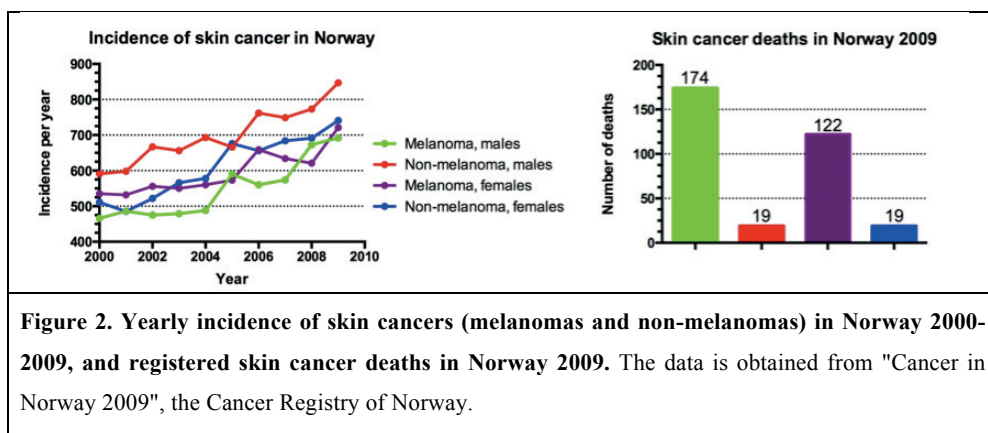
1.2.4 Brain metastasis

Epidemiology

Brain metastases are the most common intracranial tumors, ten times more common than primary brain tumors[30,57] and occurs in 15-40% of all cancer patients[58]. The yearly incidence in the US is around 170 000[59,60]. The frequency of brain metastases appears to increase, which is likely due to longer survival caused by better treatment of the primary cancer[58], and improvements in detection of smaller lesions using more advanced imaging techniques[61]. Once a patient is diagnosed with brain metastasis the prognosis is poor. The median survival of untreated patients is 1-2 months, while chemotherapy in conjunction with surgery and radiation treatment extends the survival to 4-6 months[62,63]. The poor prognosis is primarily due to resistance to chemotherapy, and recurrent growth at the site of resected lesions as well as in other parts of the brain[64].

The most common primary cancer that metastasizes to the brain is lung cancer (in 9.7-64% of the patients), followed by breast cancer (in 2-25% of the patients) and malignant melanoma (in 4-20% of the patients)[65]. Metastases to the brain from cancers of the colorectal, genitourinary tract and sarcomas are rare (1%). In up to 15% of all patients with brain metastasis, the primary cancer is unknown[58]. Brain metastases from melanomas are usually multi-focal, and sometimes associated with hemorrhage[66].

Brain metastasis: Special considerations regarding extravasation and colonization

Circulating tumor cells are brought to the brain through blood vessels, as the brain itself do not contain lymphatic vessels. It has been shown that the distribution of brain metastases correlate with blood flow and tissue volume. 80% of the tumors are detected in the cerebral hemispheres, 15% in the cerebellum and 5% in the brain stem[67].

When entering the brain circulatory system, tumor cells may be trapped in areas of slow blood flow, such as vascular branch points[68,69]. The arrested tumor cells

interact with luminal endothelial cells, that may promote tumor cell growth and invasion[69,70]. The tumor cells attach to the endothelial cells, followed by penetration through the blood-brain barrier (BBB) (Fig. 3). The structure of BBB consists of continuous, non-fenestrated endothelial cells connected with tight junctions. The outside part of the vessels is covered by a basement membrane, astrocytic endfeet and occasionally pericytes. This limits the entrance of macromolecules into the brain, and makes the brain an immunopriviliged site[58].
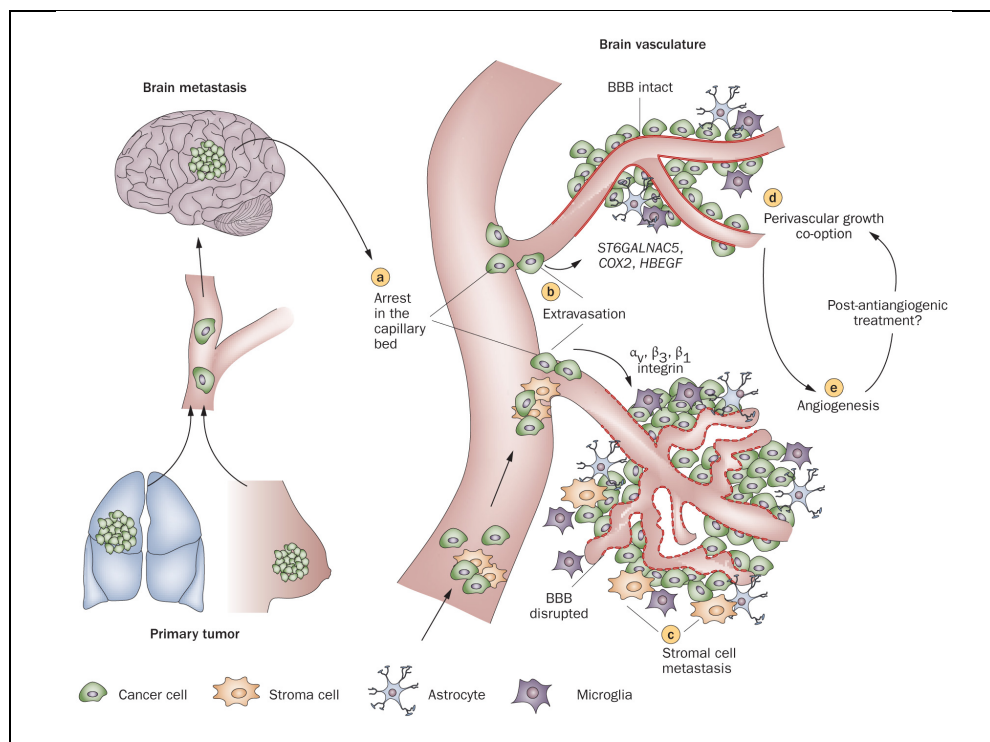
Figure 3. Steps of brain metastasis formation: Tumor cells, released from primary tumor, enter the blood stream, a) arrest in the brain capillaries, primarily due to size restrictions, and b) extravasate through the BBB and enter into the brain parenchyma. Genes involved in extravasation: ST6GALNAC5, HBEGF and COX2. Also, activation of integrins possibly control arrest of tumor cells and adhesion to endothelial cells. c) The metastatic tumor cells (the seed) may bring their own host cells (the soil). After extravasation, tumor cells may d) grow along pre-existing vessels (co-option), or e) initiate angiogenesis to obtain sufficient amount of nutrients. Adapted from Eichler et al[68]. Reprinted with permission from the publisher.

Angiogenesis. For metastases to proliferate and grow further, blood supply is necessary. Blood supply provides the tumor tissue with oxygen, growth factors, nutrients and metabolites. The process of formation of new blood vessels from existing blood vessels is termed angiogenesis. This process is switched on when the balance between the angiogenic inducing factors and endogenous factors inhibiting angiogenesis goes in the favor of the inducing factors[71,72].

Angiogenesis is a complex process involving multiple factors that stimulate the endothelial cells. The most prominent are the vascular endothelial growth factor (VEGF), angiopoietin, ephrin (Eph), platelet-derived growth factor (PDGF), transforming growth factor (TGF-β) and basic fibroblast growth factor (bFGF) families. VEGF also stimulates progenitor endothelial cells and the pericytes that lines the mature endothelial vessels[31]. VEGFR and VEGF have been shown to influence the survival, proliferation and invasion properties of tumor cell lines through the Erk 1/2 and PI3K signaling pathways [73,74].

The brain microenvironment. Cancer cells penetrating into the brain tissue encounter different host cell types, including astrocytes and microglia. Cancer cell arrest, extravasation and invasion in brain tissue have been shown to induce strong local activation of astrocytes (up-regulation of GFAP, nestin, and occasionally MMP-9), and activation of microglia to varying degrees[75]. *In vitro* co-cultures have also shown that glia can induce a fivefold increase in metastatic cell proliferation[76], indicating that reactive glia cells may change the brain microenvironment to be more permissive to tumor cell growth and development.

Astrocytes may also serve to protect the brain metastatic cells from cytotoxic effects of chemotherapy. It has been shown *in vitro* that when melanoma brain metastasis cells were co-cultured with astrocytes, a reduced apoptosis in the tumor cells was observed after treatment with paclitaxel, cisplatin and 5-fluorouracil. This chemo-protective effect was dependent on a direct contact between the two cell types[62].

It has further been shown experimentally that stromal cells from the primary neoplasm such as fibroblasts are found within brain metastasis from carcinomas,

suggesting a role of fibroblasts in metastatic colonization of the brain[77]. The stromal cells likely provide survival and proliferative advantages to the tumor cells and facilitate early colonization steps[68].

Genetic factors likely to play a role in brain metastasis

Several studies from the clinic as well as from utilizing animal models have described putative molecular mechanisms involved in the metastatic process. For instance, HBEGF, COX2 and ST6GALNAC5 are genes that may be involved in mediating the migration of tumor cells across the BBB[78]. Integrins (such as α_v, β_1 and β_3) are important for sprouting endothelial cells and may thus play an important role in angiogenesis (Fig. 3). In particular, activation of $\alpha_v\beta_3$ may enable the tumor cells to attract blood vessels through up-regulation of VEGF, independent of hypoxia[79] (see Paper I for a more detailed discussion on gene signatures associated with the metastatic process).

Models of brain metastasis

Substantial progress has been made during the last decades to develop representative animal model for brain metastases. Either rodent syngeneic models or human-rodent xenotransplantation models have been widely used (see Paper I for a thorough discussion). Currently few metastatic model systems exist, where human tumor tissue is xenografted orthotopically[68]. Current orthotopic models show systemic disease before brain metastasis occurs, thus necessitating hematogenous dissemination of tumor cells to the brain (for instance by intracardiac cell injections). Such models do therefore not recapitulate all steps of the metastatic process, as they miss the initial steps of the metastatic cascade.

Imaging of brain metastases

In preclinical brain metastatic research, *in vivo* imaging is indispensable when assessing tumor development and treatment responses. The imaging methods are non-invasive, thus the same animals can be studied several times during metastatic development. Multimodal imaging approaches are usually necessary in preclinical

studies, as the various modalities give different answers to anatomical and physiological questions.

Several imaging techniques used in the clinic have been redesigned for use in animals, such as computerized tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission tomography (SPECT) and ultrasound (US) imaging. Other imaging techniques initially used for *in vitro* studies have been redesigned for *in vivo* experiments, such as nuclear magnetic resonance (NMR), bioluminescence imaging (BLI) and fluorescence imaging (FLI)[80,81].

This chapter focuses on four of the most commonly used preclinical imaging techniques (MRI, PET, BLI and CT). For a comprehensive review on all molecular and cellular imaging methods, see for instance Lucignani and colleagues[80], while an overview of available clinical imaging strategies is given for instance by Bruno Morgan[82].

Magnetic resonance imaging (MRI). MRI makes use of the fact that body tissue contains lots of water, and hence protons. When an object is inside the strong, static magnetic field of the MR machine, all protons become aligned with the direction of the field. A radio frequency current is briefly turned on, producing a varying electromagnetic field which forces the proton spins to flip out from this direction. After the electromagnetic field is turned off, the spins of the protons return back to their original position, and the protons become re-aligned with the static magnetic field. During this relaxation, a radio frequency signal is generated from the protons, which can be measured with receiver coils, and 2D or 3D images can be generated by advanced mathematical calculation methods[80,81].
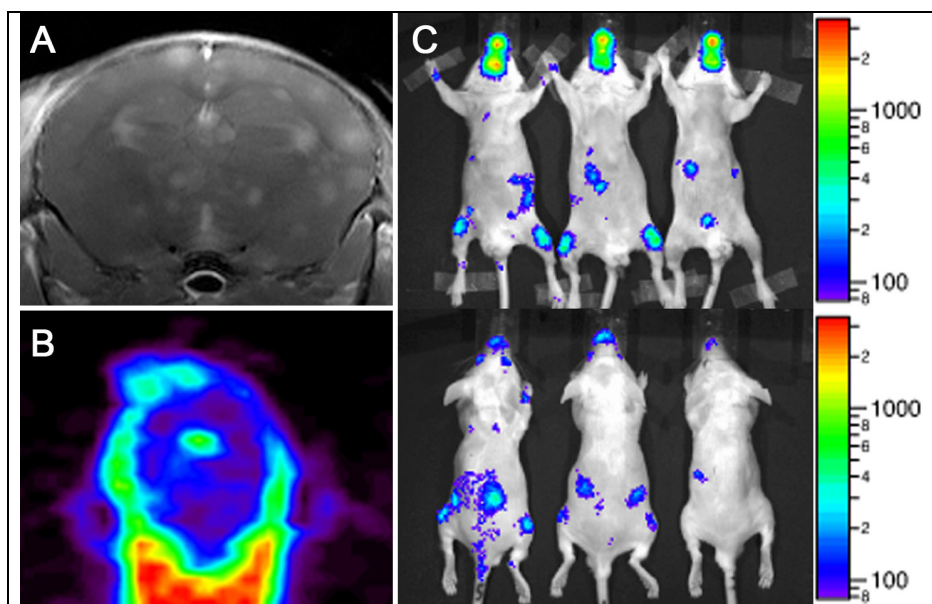
Figure 4. Examples of images obtained by different preclinical imaging modalities. A) Axial T1 weighted MR image, using Prohance contrast agent, of metastatic melanoma spread in the mouse brain, 6 weeks after ICD injections of tumor cells. Multiple, small contrast-enhancing tumors are seen. B) Coronal ^{18}F-FLT PET image of a tumor-bearing mouse 6 weeks after tumor cell injection. A central area with proliferating tumor cells is seen. C) Bioluminescence images of ventral side (upper) and dorsal side (lower) of 3 mice, 2 weeks after tumor cell injections. Tumor spread is detected in the brain as well as in other organs of the animals (all pictures by courtesy of Frits Thorsen).

MR imaging is crucial in preclinical brain metastatic research, due to excellent soft tissue contrast and high resolution[83] (Fig. 4A). MR contrast agents have commonly been applied prior to imaging, to study metastatic spread of single breast cancer cells and melanoma cells to the brain[84,85] (Paper III), or to investigate development of solid brain metastasis from melanoma[86] (Paper III) or breast cancer[87].

Positron emission tomography (PET). PET is a nuclear medicine imaging technique that produces a three-dimensional image of functional processes in the body. The system detects pairs of gamma rays emitted indirectly by a positron-emitting radionuclide (tracer), which is introduced into the body on a biologically

active molecule[88]. Three-dimensional images of tracer concentration within the body are then constructed by computer analysis. PET scanning is usually accomplished with the aid of CT scanning, to align the functional data from PET with anatomical information. A number of different PET tracers are available for cancer monitoring. The most widely used tracer is the glucose analogue 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) which measure glucose metabolism, further 3´-[^{18}F]fluoro-3´-deoxythimidine ([^{18}F]FLT) is used for monitoring tumor cell proliferation[89] (Figure 4B). A detailed discussion of available PET tracers for oncology is beyond the scope of this thesis.

PET imaging has been used in preclinical models of primary malignant brain tumors, for instance to determine tumor activity in different glioblastoma phenotypes implanted into the brains of immunodeficient rats[90]. However, very little is published on PET imaging of brain metastasis models.

Bioluminescence imaging (BLI). A BLI signal is the result of an enzymatic reaction where luciferase catalyzes the oxygenation of luciferin using ATP and molecular oxygen to yield oxyluciferin. This enzyme converts chemical energy into photon energy, resulting in a measurable emission of light. The luciferase gene is commonly stably transfected into the cancer cells by viral vectors, to visualize tumor growth and development of the tumor cells in mouse models[91]. Luciferase-transfected tumor cells are injected into the mice, either orthotopically[78] or via the bloodstream (Paper III). After a certain period of tumor growth (usually a few days), luciferin substrate is injected into the mice, and the enzymatic reaction with luciferase will result in detectable light in areas of tumor growth, which will be revealed by the resulting bioluminescent image (Fig. 4C).

Bioluminescence imaging has commonly been used in preclinical experiments, to study systemic spread of tumor cells as well as specific tumor cell spread to the brain[78,92,93] (Paper II and III).

Computed tomography (CT). In CT, a fan-beam of X-rays is attenuated or absorbed during passage through the body. This results in difference in attenuation in different tissues, which is detected and calculated into 2D images. CT has in general

poor soft tissue contrast, but this can to some extent be overcome by distributing contrast agents to the body. The technique cannot provide information on tissue biochemistry and physiology. The imaging technique is however excellent for studying for instance metastasis to bone. Due to very high spatial resolution, CT is often used to give anatomical reference images that are co-registered with functional imaging, such as for instance PET[88,94].

1.3 Therapeutic strategies for brain metastases

Patients with brain metastases that are left untreated have a poor prognosis, with an estimated survival of 1-2 months[62,63,95]. Therefore treatment for brain metastases is necessary. As the treatment varies widely, a sound prognostic index is important for guidance in making the clinical decision.

Prognostic Factors

A Radiation Therapy Oncology Group (RTOG) study reviewed about 1200 patients enrolled in clinical trials that used Whole Brain Radiation Therapy (WBRT), and analyzed prognostic factors by recursive-partitioning analysis[a] (RPA) classes I-III[59]. Favorable prognostic factors for patients with brain metastases were Karnofsky performance status[b] (KPS) of 70 or more, representing Class I (accounting for 20% of all subjects). Patients in this class had the following criteria: no distant metastasis other than brain metastases, controlled primary tumor, and age less than 65 years. KPS less than 70 was a poor prognostic factor and characterized as class III (accounting for 15%), whereas all other cases were considered to represent class II (accounting for 65%). The median survival rates were 7.1, 4.2 and 2.3 months for patients in RPA class I, II, and III respectively (Table 2).

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[a] Recursive partitioning is a statistical method for multivariable analysis.
[b] The Karnofsky performance scale is an assessment tool intended to assist clinicians and caretakers in gauging a patient´s functional status and ability to carry out activities of daily living.

RPA Class	Prognostic Factors	Median survival (months)			
		WBRT[59] (n=1176)	SRS[96] (n=265)	WBRT+SRS[96] (n=295)	Surgery+WBRT[97] (n=125)
I	KPS ≥70, age <65, Controlled primary tumor, No extra-cranial metastases	7.1	14.0	15.2	14.8
II	KPS ≥70 but other than class I	4.2	8.2	7.0	9.9
III	KPS <70	2.3	5.3	5.5	6.0

Table 2: Recursive portioning analysis (RPA) classification and prognoses of patients with brain metastases. KPS (Karnofsky performance status); WBRT (Whole brain radiotherapy); SRS (Stereotactic radiosurgery). (References 59, 97-98)

A more recent review of the RTOG database has resulted in new prognostic classification called Graded Prognostic Assessment (GPA), which incorporates the number of intracranial metastases to already stated prognostic factors[98]. Median survival ranges from 2.6 months in the poorest prognostic group (age >60, KPS <70, >3 intracranial metastases and extracranial metastases present) to 11 months in the most favorable prognostic group (age <50, KPS 90-100, 1 intracranial metastasis and no extracranial metastases).

Therapeutic Strategies

Therapeutic strategies of brain metastases may be divided into two groups: Palliative Therapy, aimed at reducing symptoms; and Definitive Therapy, aimed at reducing tumor burden [99,100](Table 3).

Palliative Therapy	Definitive Therapy
Corticosteroids	Surgery
Anticonvulsants	Chemotherapy
	Whole-brain Radiotherapy (WBRT)
	Stereotactic Radiosurgery (SRS)
	Radiosensitizers
	Immunotherapy
	Combination of above stated therapies

Table 3: Available treatment options for patients with brain metastases.

These two groups will be discussed briefly as follows:

1.3.1 Palliative Therapy

Palliative therapy (Symptomatic treatment) focuses mainly on reducing tumor related symptoms, thus increasing the quality of life rather than increasing survival.

Corticosteroids

Corticosteroid is commonly the drug of choice for the patients with increased intracranial pressure secondary to the total intracranial tumor burden and vasogenic edema. Dexamethasone is used because of its low mineral-corticoid effect and it rapidly relieves the peritumoral edema. However, dexamethasone can impair the penetration of the chemotherapeutic drugs into the brain tumor or surrounding tissue, therefore simultaneous use of corticosteroids and chemotherapy should be individually evaluated[101,102].

Anticonvulsants

Epileptic seizures are triggered by brain metastases in 25-40% of the cases[103], which necessitates anticonvulsant therapy. Levetiracetam shows superior efficacy and/or tolerability as compared to other anticonvulsants[104]. In the absence of any

history of epileptic seizures, prophylactic antiepileptic treatment is usually justified for 2-6 months after surgical excision of cerebral metastases[105].

1.3.2 Definitive or Specific Therapy

The intention with definite therapy is to restore neurological function, remove tumor burden and extend patient survival[106]. A therapeutic strategy that may be used alone or in combination includes surgery, chemotherapy, WBRT, SRS, radiosensitizers and immunotherapy. The choice of Definitive therapy depends upon many factors such as the number, size and location of brain tumors as well as histology of the primary tumor and extent of systemic extracranial disease[107-109].

Patients with brain metastases often have a highly progressing disease, which necessitates rapid determination of the therapeutic strategy. A decision tree (Fig. 5) may help to determine which type of treatment each individual patient with brain metastasis needs[109,110].

Surgery

Surgery has an indispensible role in the management of brain metastases[107]. With the widespread availability and improved imaging modalities such as MR imaging and CT imaging, surgical resection has become more feasible. Also advances in neurosurgical techniques over the past two decades have resulted in safe practice and decreased rate of surgical mortality and morbidity[111].
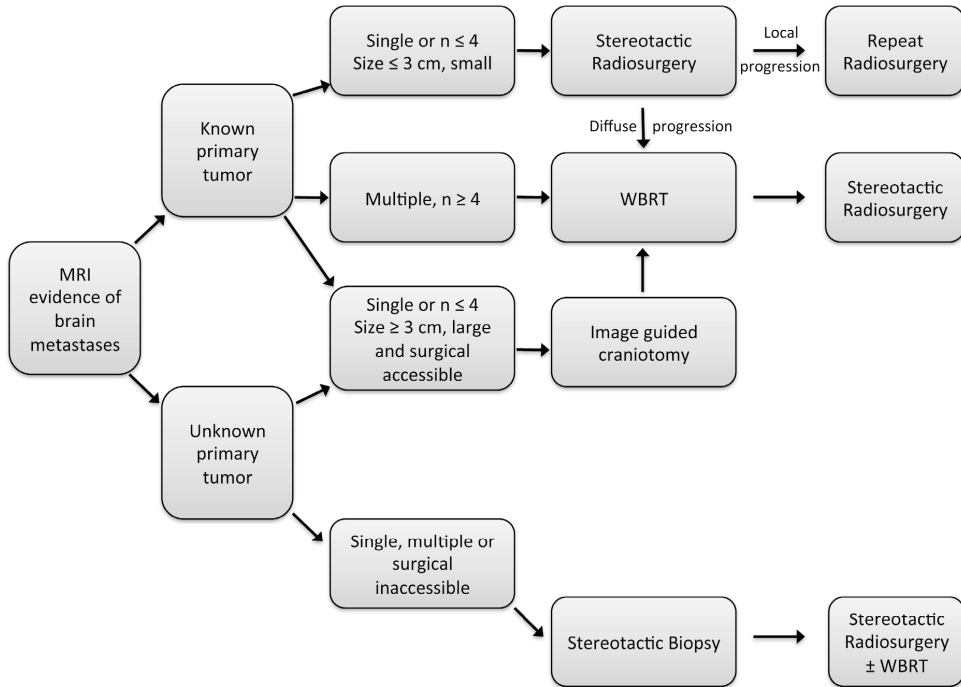
Figure 5: Decision tree for treatment of patients with brain metastases (modified from Sheehan et al[110] and Narita et al[109])

Criteria for selection of patients for surgical resection:

There are three factors that should be carefully considered before surgical resection of brain metastases in patients. These are clinical and functional status of the patient (Karnofsky Performance Status), the histology and grade of the metastatic lesion and number, and the size and location of lesions in the brain[107]. Surgery should be considered for patients with good performance status, stable extracranial disease and lesion size of 3 cm or greater in diameter. Studies have recognized the importance of surgery, not only for the patients with single brain metastasis but also in the case of multiple brain metastases. It has been shown in selected patients with multiple brain metastases, stable systemic disease and good performance status that surgical removal of all lesions resulted in significantly increased survival time similar to that of patients undergoing surgery for a single metastasis[112]. Even removal of a

few selected symptomatic lesions in the patients with multiple brain metastases, may result in better survival and symptomatic relief[113].

Studies done by Patchell and others[114,115] have shown that for solitary brain metastasis, surgical resection should be the initial standard treatment complemented with postoperative WBRT. Surgical resection without post-operative WBRT resulted in a 15% risk of a local reoccurrence independently of the origin of the primary cancer site. Postoperative WBRT is expected to destroy the microscopic residual cancer cells at the site of resection as well as in other brain locations, if they exist, thereby reducing the recurrence rate and prolonging survival[116].

Primary tumors can vary remarkably in their sensitivity for WBRT or chemotherapy and this may have influence on the effect of these therapies on brain metastases[117]. In that case surgical resection is almost always favorable in the patients with resectable metastases of unknown histological type.

Chemotherapy

Chemotherapy has generally been used on patients who have failed other treatment options. The results have been disappointing, due to the inability of many drugs to cross the BBB, and due to the insensitivity of the tumor cells to the particular drug[106].

The response rate of the metastatic lesions frequently correlates with the sensitivity of the primary tumor to chemotherapy[119,120]. Thus the choice of chemotherapeutic regimen should depend more on the tumor histology rather than only on the distribution of the single drug in the brain. Response rates for chemotherapy are relatively higher in small cell lung cancer (30-80%), intermediate in breast cancer (30-50%) and non-small cell lung cancer (10-30%) and low in melanoma (10-15%)[120-122]. It has also been hypothesized that brain metastases from primary tumors with intrinsically low levels of P-glycoprotein expression (a protein linked to chemoresistance) in tumor vessels may be more permeable to chemotherapeutic drugs[123].

Further chemotherapy for malignant melanoma will be discussed briefly. An overview of completed and ongoing clinical trials on malignant melanoma brain metastases is shown in Table 4.

Drug Name	Target/s	Patient Enrollment (n)	Study Phase	Study status	Relevant References
PLX4032 (Vemurafenib)	BRAFV600E	132	II	Active	Keating et al[124]
Temozolomide	Methylating agent, DNA ligase IV	162	II	Completed	Siena et al[125]
RO5185426	BRAFV600E	24	II	Completed	Sosman et al[126]
Ipilimumab	CTLA-4 receptor	72	II	Active	Margolin et al[127]
GSK2118436	BRAFV600E	172	II	Active	Ribas et al[128]
Temozolomide and Thalidomide	DNA ligase IV and Sedative		II	Completed	Krown et al[129]; Hwu et al[130]
Temozolomide, Thalidomide and Lomustine (TTL)	DNA Ligase IV; Sedative; Alkylating agent	17	II	Completed	Quirbt et al[131]
Bevacizumab, Dacarbazine and Interferon-Alfa-2a	VEGF-A; Alkylating agent; Immune cells	27	II	Completed	Vihinen et al[132]
Temozolomide and Sorafenib	DNA Ligase IV; Kinases (C-Raf, B-Raf, VEGF-2, -3, PDGF, Flt-3, c-Kit)	167	II	Unknown	Amaravadi et al[133]
Abraxane; Temozolomide; Genasense® (Oblimersen)	Enhancing tubulin polymerization and suppressing spindle microtubule dynamics; DNA Ligase IV; Targets first six codes of Bcl-2 mRNA	162	II	Completed	Miele et al[134,135]
Lomustine; Cytarabine: Radiotherapy	Alkylating agent; DNA synthesis	9	I	Active	Koller et al[136]; Dueland et al[137]

Monoclonal antibody Me 1-14 F (ab´) 2	Glycoproteins	6	I	Unknown	Zalutsky et al[138]
Boronophenylalanine-fructose complex (Radiochemotherapy)	DNA (amino acid selectively accumulate in melanoma cells by mimicking Phenylalanine)	Not stated	I/II	Unknown	Kiger et al[139]; Liberman et al[140]
Temozolomide; Bevacizumab	DNA Ligase IV; VEGF-E	34	II	Completed	Von Moos et al[141]
Temozolomide plus Radiation Therapy	DNA Ligase IV	41	II	Completed	Kouvaris et al[142]; Margolin et al[143]
Disulfiram	Target through ROS production and activate extrinsic pathway of apoptosis	7	I/II	Completed	Morrison et al[144]

Table 4: Recent ongoing and completed clinical trials on malignant melanoma brain metastases from ClinicalTrials.gov.

Dacarbazine (DTIC) is an alkylating agent, and the first drug approved by the FDA for treatment of metastatic melanoma. It has been used as a single-agent standard therapy for 3 decades, with response rates of 8-20% with an average duration of response of approx. 4-6 months. The limited response to DTIC is presumably due to a low penetration efficacy through the CNS[145].

Temozolomide (TMZ), an oral analog of DTIC on the other hand, penetrates the BBB. Clinical studies on TMZ have described complete regression of multiple brain metastases from melanoma[146,147]. Paul and colleagues[148] demonstrated that a replacement of DTIC with TMZ might in fact reduce the incidence of CNS lesions in patients. In this study, with a median follow-up of patients for 19 months, CNS relapse occurred in 43% after DTIC therapy and 10 % after TMZ therapy. TMZ alone or in combination with other chemotherapeutic agents have been tried in several early phase clinical trials showing modest results, with an overall response rate of 4-13%[125,149,150]. TMZ has also been combined with WBRT for the treatment of established brain metastases[143,151,152], but the results have been dismal. Further,

clinical studies have shown that while TMZ fails on established brain metastases, the drug can be effective as an adjuvant treatment of microscopic intracranial metastases in non-small cell lung cancer[153]. In melanoma brain metastases, studies have shown limited anti-tumor activity with TMZ in combination with WBRT[143]. The effect of TZM and its combinations with other chemotherapeutic drugs, anti-angiogenic drugs, immunotherapy and radiotherapy are still under intense investigation in melanoma brain metastases (Table 4) and other brain metastatic diseases.

Several other chemotherapeutic drugs have been tested alone or in combination on melanoma brain metastases. Some of them are Fotemustine (Nitrisourea alkylating agent)[154], Docetaxel (anti-mitotic chemotherapy)[155] and Thalidomide (an oral anti-angiogenic agent)[130].

Whole Brain Radiotherapy (WBRT)

In contrast to surgery, radiation therapy can be delivered to the patients with relatively low morbidity. Radiation therapy has been the cornerstone of brain metastases treatment for 5 decades. Radiation has originally been regarded as a palliative treatment, intended to relieve symptoms and to lesser degree contribute to improved patient survival[106].

WBRT is considered to be the mainstay for patients with multiple brain metastases, where the primary tumors are radiosensitive. Melanoma, renal cell carcinoma and sarcoma are considered being radioresistant and thus WBRT has little influence on the overall survival and progression free survival rate. WBRT may be omitted initially in these cases and could be considered at recurrence[99,117]. On the other hand, WBRT is the treatment of choice in the patients where single or multiple brain lesions that are not amenable to surgery or radiosurgery. The median survival in this patient group range from 3-6 months with 10-15% of patients alive after 1 year[156].

Several large-scale multi-institutional trials conducted by RTOG have demonstrated that there is no significant difference in the frequency and duration of response for total radiation doses ranging from 20 Gy over one week to 50 Gy over

four weeks[157,158]. Currently, typical radiation treatment schedules consist of short courses, from 7-15 days, of WBRT with relatively high doses per fraction (1.5-4 Gy) with total doses in the range of 30-50 Gy. This minimizes the duration of treatment but still delivering the adequate dose to the tumor.

There are several complications associated with radiotherapy, such as leukoencephalopathy, progressive dementia, ataxia, and incontinence due to radiation-induced necrosis, occurring in approximately 10% of patients[159,160].

Combining WBRT with chemotherapy may have significant increase in overall survival in some brain metastases (already discussed in the chemotherapy section). Also combining WBRT with stereotactic radiosurgery has shown advantages as compared to WBRT alone in some brain metastases (discussed ahead).

Stereotactic Radiosurgery (SRS)

Stereotactic radiosurgery (SRS) is a method that utilizes multiple convergent radiation beams to deliver a high single dose of radiation to a well-circumscribed brain lesion. SRS may be performed using high energy X-rays by a linear accelerator (LINAC)[161,162], the CyberKnife system[163], or by using gamma radiation from ^{60}Co sources in the Gamma Knife[117,164]. The latter system is most commonly used, and has shown a tremendous success in treating circumscribed brain metastasis less than around 3 cm in diameter[108,165].

It is preferred for the patients with radioresistant brain metastases and/or unresectable brain metastases. SRS represents a minimally invasive technique, capable of treating multiple metastases in one setting. Prognostic factors for SRS are the KPS score, total intracranial volume and the presence of active systemic disease[166].

SRS have proven to be an effective and safe treatment option for brain metastases from all kinds of primary tumors, including radioresistant melanomas, renal cell carcinomas and sarcomas[117], where median survival is reported to range from 7-8 months[167-169]. Further for other brain metastases, the survival ranged from 6

months for patients with colon carcinoma and unknown primary tumors, to 17 month for those of breast cancer[166].

The SRS dose depends upon the shape, position and size of the lesion. Reported optimal dose to the tumor margin ranges from 15-22 Gy, with a median of 20 Gy[167]. The RTOG recommends the maximum tolerated marginal dose as 24 Gy for tumors that are less than or equal to 20 mm in diameter, 18 Gy for 21-30 mm and 15 Gy for 31-40 mm in diameter[170,171]. The rapid dose falloff minimizes the risk of damage to the surrounding normal nervous tissue. For example, in Gamma Knife treatment, only 1/201th of the total radiation dose passes through the body on the way to the target site because there are 201 converging beams and the highest dose is deposited where the 201 beams converge.

The role of SRS and SRS plus WBRT in multiple brain metastases has been controversial. RTOG demonstrated longer survival and overall KPS score improvement in patients with single unresectable brain metastasis treated with SRS and WBRT than treated with WBRT alone[172]. They also considered the combination therapy for 2-3 brain metastases but recommended combination therapy as a standard therapy for single unresectable brain metastasis.

Radiosensitizers

The ability of radiation therapy to eradicate malignant cells depends upon the intratumoral content of molecular oxygen, a potent radiosensitizer involved in mediating DNA damage[173,174]. Generally tumors consist of regions with a large number of hypoxic cells and these cells are 2-3 times more resistant to ionizing radiations as compared to cells with normal levels of oxygen[175,176]. Radiosensitizers are electron-affinic drugs, which mimics the action of oxygen but are more slowly metabolized. Thus radiosensitizers make tumor cells more sensitive to radiation therapy, which in turn may improve local tumor control[177,178].

Several radiosensitizers have been tried in clinical trials, such as Misonidazole, halogenated pyrimidine bromodeoxyuridine (BUdR), Motexafin gadolinium (MGd) and RSR13 (efeproxiral). Radiosensitizers have been not shown a success in the

treatment of melanoma brain metastases[179,180]. However, MGd with WBRT as a combination treatment showed significantly favorable results in lung cancer patients as compared to WBRT treatment alone, increasing survival to 5.5 months in the group receiving WBRT plus MGd, as compared to 3.7 months in the group receiving WBRT alone [181]. Also, RSR13 in combination with supplemental oxygen and WBRT (30 Gy in 10 fractions) has shown some promising results for breast cancer brain metastases patients with increased median survival time to 8.67 months, as compared to 4.57 months in patients treated without RSR13[182].

Immunotherapy

Immunotherapy towards brain metastases includes induction or enhancement of the immune response. For instance, interferon-α (IFN-α) up-regulates major histocompatibility complex antigen processing and co-stimulatory molecules, which leads to more efficient antigen presentation that may induce auto-reactive activity of T-cells[183,184]. This may lead to a potent anti-tumor cell-mediated cytotoxicity[185].

Some responses to immunotherapy have been studied in melanoma brain metastasis by combining biological response modifiers (BRMs) and cellular immunotherapy. For instance, Savas and colleagues observed near complete response of brain metastases, unresponsive to radiation therapy, after treatment with a regimen consisting of interleukin-2 (IL-2), interferon (IFN), and 5-fluorouracil[186], (see also Table 4 for some recent trials in this field). Immunotherapy in combination with chemotherapy is also under clinical trials[187].

2. Aims of the study

1. To review the literature and point at the advantages and disadvantages of the various brain metastasis models that have been developed.

2. To develop an orthotopic rat model to study the *in situ* growth and progression of brain metastasis.

3. To develop a reproducible and robust brain metastasis model for human melanoma, by performing MR imaging of single, prelabeled cells in the mouse brains, with subsequent automated cell detection and quantification.

4. To assess the effect of combination therapy on melanoma brain metastatic cells *in vitro* by targeting the MAPK and PI3K pathways.

3. Methodological considerations

Patient biopsy material collection

In this thesis, fresh resected patient brain metastases were obtained during surgery, from the Department of Neurosurgery, Haukeland University Hospital, Bergen. All collection of patient tumor tissue was carried out after written consent from patients before surgery. The regional ethical committee (#013.09) and the Norwegian Directorate of Health (#9634) approved the tissue collection and storage in a biobank.

Animal models

All the animal experimental protocols and procedures used in the study were approved by and performed according to regulations of the National Animal Research Authority.

Reliable and reproducible animal models are crucial in order to understand complex processes of tumor growth and progression including metastasis. As discussed in Paper I, different animal brain metastasis models are available with their advantages and limitations. In paper II, we developed a clinically relevant orthotopic animal brain metastasis model showing similar growth characteristics as the parental brain metastases. The development of this model system used in this study was based on prior experiences with establishing animal glioblastoma models[188,189,200]. Fresh patient brain metastases tissue, obtained from surgery was minced and transferred to agar-coated culture flasks containing standard tissue culture serum-supplemented medium. Multicellular aggregates (spheroids) were formed after 2-4 weeks, which were then implanted into the nude rats brains. DNA copy number profiles, histology, immunohistochemistry and MRI showed strong similarities between animal xenografts and the parental tumors.

Initially patient brain metastases from different primary tumors were screened to see if they were able to develop tumors in animal brains. Out of the nine, seven metastases showed tumor take in animal brains. These established tumors were then

removed, cultured again into spheroids, and thereafter serially passaged into new nude rat brains with 100% tumor take.

From a patient melanoma brain metastasis, we developed a cell line named H1. H1 cells were injected in the skin of immunodeficient NOD SCID mice (data not published), which are deficient in both T- and B-cells[190,191]. The intradermal injections led to a 100% tumor take, yet the cells did not invade and failed to form distant metastases in brain or other organs of the animals. Thus in paper II and III, we chose to introduce H1 cells directly into the circulation by ICD inoculation, to mimic the escaped circulating tumor cells excluding the initial step of tumor cell invasion and intravasation into the blood vasculature[193]. This led to the development of metastases in different organs including brain. The animals were followed regularly by BLI.

We had approximately 90% success rate using free hand for ICD injections. We chose ICD injection for tumor cell inoculation as the technique is easy to perform compared to intracarotid (ICA) injections, which needs expertise to do the ligation surgery and also IV injections were excluded due to limitation of tumor cells entrapment into the lungs after injection.

Magnetic Resonance Imaging (MRI)

To study longitudinal development of brain metastasis in our animal model, MRI was chosen because of its high spatial resolution and excellent soft tissue contrast. A 7 Tesla small animal MR scanner equipped with a circular mouse head transmit/receive coil (Bruker Biospin MRI GmbH, Germany) was used for all our studies.

In paper II, T1 weighted MRI before and after intraperitoneal injection of 0.1 mL Omniscan contrast agent, (0.5 mmol/ml; GE Healthcare, Norway) was used to study tumor development in the mouse brain, and the post contrast images showed tumor enhancement. T2 weighted MR imaging of the animal brains showed increased edema, necrotic tumor areas and midline shift due to progressive growth of lesions. In

paper III, T2* weighted MR images were obtained to visualize SPION positive particles (see below).

Superparamagnetic iron oxide nanoparticles (SPIONs) and quantification of SPIONs

To track the melanoma brain metastases cells immediately after intracardial injection and to prove successful injections, the cells were prelabeled with superparamagnetic iron oxide nanoparticles (SPIONs) for T2* weighted MRI. Iron oxide is the main component of SPIONs, which induces large changes in the magnetic susceptibility, which in turn lead to hypointensities in T2* weighted images. Due to the large magnetic susceptibility of an iron oxide particle, the loss of signal is usually much larger than the particle size, which in turn enhances detectability at the expenses of resolution. The SPION particles have shown to exhibit low toxicity, and may be recycled by cells via natural metabolic pathways[194,195].

The nanoparticles used in our study were, maghemite particles coated with poly-L-Lysine (named PLL-γ-Fe$_2$O$_3$), with a diameter less than 100 nm[196]. The polymer coating of poly-L-Lysine makes the nanoparticles more stable, specific and efficient in internalization into the target cells than other type of coated SPIONs[197,198].

Many brain metastasis animal models are based on intracardial injections of tumor cells. However, due to the difficulties in keeping the needle tip stably inside the left cardiac ventricle during injection, none of the models technically assure adequate delivery of the same amount of tumor cells to the brain. Therefore, reliable and standardized therapeutic results may be hard to achieve[199]. In our melanoma brain metastasis model, tumor cells prelabeled with SPIONs were injected into the left cardiac ventricle, followed by T2* weighted MRI after 24 hours. The tumor cells appeared as hypointensive spots on the T2* weighted images, and the number of cells were quantified by fully automated analysis software developed in MATLAB 7.14 (MathWorks) (For details, see paper III). In this way, injection failures could be

excluded from our animal experiments and more reliable experimental results could be achieved.

DNA copy number analysis

We used Affymetrix 250K SNP array to study DNA copy number variations in patient brain metastases before and after implantation in our orthotopic brain metastases animal model (paper II). This analysis showed strong similarities in the genomic profiles of patient brain metastases and the respective xenografts from the animal model. This indicated that the tumor maintained the same biological phenotype in our model as observed in the patients.

4. Results and Discussion

Paper I

As outlined in paper I, several brain metastasis models have been developed during the last 60 years. However, based on current information, it is evident that none of the models developed fully reflect all aspects of the metastasis process in humans. Still, these model systems have provided important insight into specific mechanisms of the brain metastatic process.

Brain metastatic animal models may be divided into 2 broad groups, rodent syngeneic models, and human-rodent xenotransplantation models.

Rodent syngeneic models use murine derived cell lines, and can be divided into 2 groups: ectopic injection and orthotopic injection. Genetically engineered mouse models (GEMMs) may be regarded as a subclass of orthotopic rodent syngeneic models, since genetic manipulations in mice result in the development of primary malignancies, followed by metastasis to other organs, including brain. Advantages with syngeneic brain metastasis models are that the tumor development occurs in immunocompetent mice and there is relative short latency period between injections and metastatic spread.

Human-rodent xenotransplantation models use immunocompromised animals and are further divided into 2 groups: ectopic injection and orthotopic injection. In orthotopic animal models, tumor cells are injected in the same organ of the animals as the origin of the corresponding human tumor. Such models have provided insight into the metastatic process, for instance in studies of tumor self-seeding processes. However, established cell lines have undergone clonal selection, and genotypic and phenotypic alterations that make them different from the tumor of origin. Thus validation of such models against clinical brain metastases is mandatory.

A variant of the orthotopic brain metastases models, where cells or biopsies from patient brain metastases are implanted directly into the animal brain, should be regarded as growth models, as they represent only the final step of the metastatic process. Yet, these models can be used to study molecular mechanisms responsible

for solid metastasis development and for the development of new local therapies. A major advantage using such models is that the human brain metastatic tissue also contains stromal elements, where clonal selection, to a large extent is avoided as verified by aCGH analyses (see paper II).

Ectopic animal models have been established using various inoculation routes: Intravenous (IV), intracardial (ICD) and intracarotid (ICA) inoculations. None of these mimic all steps of the metastatic process in humans. IV injections lead to pulmonary entrapment of cells in lungs whereas ICA injections change the normal blood supply to the brain after permanent ligation of the carotid artery. Both these inoculation techniques are disadvantageous in homing studies of tumor cells to various organs. In contrast, ICD injections are preferred in homing studies but still present other limitations, as free-hand ICD injections have sometimes been found to have relatively high procedural mortality rate.

Paper II

In paper II we developed a clinically relevant orthotopic animal model, which represents the tumor growth and progression of brain metastases in patients. In this model we implanted patient brain metastases derived spheroids directly into the animal brains using the previously established intracranial implantation procedure for human glioblastomas[188,189]. Seven out of nine (77.8%) transplanted brain metastases showed tumor take. These animal brain metastases were then removed, cultured into spheroids and re-implanted into new nude rats brain resulting in a 100% tumor take rate. Brain metastases developed in the rats showed striking similarities to the human metastases with respect to DNA copy number profiles, histology, immunohistochemistry and MRI. The tumor growth represented final step of the brain metastasis process and is a valuable tool to study the growth of metastatic tumors within the CNS and to study the new therapeutic avenues for metastatic brain tumors.

The variable tumor take in the initial screening of the nine patient brain metastases could be due to an immune reaction between the nude rat brains and the

tumor tissue. Some still remaining immunocompetent cells, such as NK-cells and T-cells, in nude rats could potentially reject the establishment of the xenografts.

Interestingly, when the same tumors were injected at a sub-cutaneous location, no tumor take was observed. This indicates that the brain represents a favorable environment for tumor growth (soil). In this context it should be emphasized that the CNS represents an immuno-privileged site that may predispose tumor growth.

In summary, this orthotopic xenotranplantation model led to a successful establishment of patient brain metastases in the animal brains that to a large extent mimicked the *in situ* progression and development of brain metastases in humans.

Paper III

In this paper, our aim was to develop a robust model of brain metastasis that enabled quantitative tracking of single tumor cell dissemination and tumor progression within the CNS. We developed tumor cell lines from patient brain metastases that were stably transfected with GFP and Luciferase firefly reporter genes. To monitor dissemination to different organs, we delivered the cells by ICD injections and were able to show metastases in different organs including the brain.

It has previously been shown that the number of cells that reach the brain varies when performing ICD injections, as it is often difficult to position the needle tip steadily within the left cardiac ventricle during inoculation[201,202]. In preclinical experiments, such variations in cell number will inevitably lead to unreliable results that may be a hindrance in success of treatment experiments. Therefore we developed a robust technique, by prelabeling the cells with superparamagnetic iron oxide particles (SPIONs) prior to inoculation. This enabled us to track in detail single tumor cells within the brain using MRI. The MRI images were evaluated using image processing software and analyzed in a fully automated fashion using signal detection algorithms developed in MATLAB. SPIONs are in general considered to show good biocompatibility and low toxicity[203,204]. These particles, which display high magnetic signal strength by MRI, enabled us to sort out animals with successful ICD injections, and to quantify the number of cancer cells within the brain. This fully automated

MRI-based quantification of SPION labeled tumor cells has an advantage since it will avoid variations in preclinical therapy trials. It also allows the visualization of the actual cancer load in the brain after ICD injections. This technique is therefore important for the critical delivery of comparable cell numbers to the brain. This is important for obtaining reliable data and will increase the precision level of therapeutic preclinical studies.

The automated quantification system also had its limitations. The hypointensive spots seen in T2*weighted MR images varied in size, and we were not able to completely separate single cells from multiple tumor cells within the brain, an observation that was confirmed by immunohistochemistry. Further improvements in MR imaging techniques may improve these results, for instance by using better shimming and more optimized T2* techniques. Nonetheless, we show that the labeling technique registers tumor cell numbers, which are proportional to the injected quantities of tumor cells as well to the number of solid brain metastases formed. Thus, the fully automated quantification technique has the potential of improving therapeutic assessment in preclinical trials.

Paper IV

There is now extensive data showing the importance of the MAPK[205,206] and the PI3K[207] pathways in melanoma progression. Our data shows that the H1 cell line harbors both the BRAFV600E mutation as well as loss in PTEN, which indicates that the MAPK and the PI3K pathways are activated. In paper IV, we targeted these two pathways using two novel drugs: PLX4032, that inhibits BRAFV600E, which is part of the MAPK pathway, and Temsirolimus, that inhibits mTOR, which is a part of the PI3K pathway.

Our *in vitro* results show that targeting these two pathways simultaneously inhibited melanoma growth and proliferation as compared to monotherapies that target one pathway at a time. Previous studies have shown that the BRAFV600E inhibitor, PLX4032 rapidly developed resistance after a short term clinical benefit[208,209] and also Temsirolimus alone could not show any sufficient clinical

response in patients[210]. In our study, combined treatment showed a significantly synergistic inhibition in H1 cell proliferation and successfully inhibited H1 spheroid viability and survival; also Western blot analysis confirmed the loss of pMAPK and p-mTOR expression, along with a reduced pAKT expression. Thus our work showed a significant inhibitory effect of combined therapy over PLX4032 and Temsirolimus monotherapies.

Loss of PTEN expression causes an increase in PI3K/pAKT activity, in cases where BRAF is inhibited and contributes to intrinsic resistance of BRAFV600E mutated melanoma cell lines to PLX4032[211]. Therefore combining therapy against these two pathways is justifiable. In our study, Western blot analysis showed that combined therapy reduced the pAKT expression in the H1 cells as compared to monotherapies and indicates the inhibitory effect of combined therapy on the PI3K signaling pathway.

The gene expression profiles of H1 cells were assessed by microarray analysis before (untreated control) and after combination treatment. The results indicated important downstream functions that were affected by the combined treatment, such as cell cycle, cell-death and -survival, cell movement and DNA-replication, -regulation and -repair.

Currently, work is ongoing in our lab, using the same approach of combined therapy on human melanoma brain metastasis cells harboring the wild type BRAF gene. Our work indicates that combined treatment with PLX4032 and Temsirolimus leads to a specific effect on melanoma cells harboring the BRAFV600E mutation as compared to melanoma cells with wild type BRAF gene.

In conclusion, combined therapy of melanoma brain metastasis cells using PLX4032 and Temsirolimus shows a promising strategy and forms a solid base for future preclinical experiments.

5. Conclusions

Paper I

By recapitulating the literature on metastatic disease, none of the currently available brain metastasis animal models fully represent the metastatic process seen in humans, although some of the important underlying mechanisms in the metastatic process have been partly revealed. Genetic studies show that there is relatively little overlap between genes found to be important in animal models, and genes determined to be important in clinical studies. The scientific question(s) in focus should determine the tumor model (rodent or human) and the route of inoculation. Future work should focus on finding the appropriate animal models that reflect the human disease.

Paper II

We show that spheroids cultured from human brain metastases can be implanted into nude rat brains with high efficiency. The derived rat brain tumors exhibited radiological, histological, immunohistochemical and genomic traits similar to human brain metastases. We were successful in serially passaging the tumor material into new animals, and thus we were able to standardize the tumor model, by achieving a 100% tumor take rate in the animals. This model may thus be an important tool to assess responses to new treatment modalities and for studying biological mechanisms causing metastatic growth in the brain.

Paper III

We developed a robust and reproducible model system for brain metastasis, where the tumor cells were prelabeled with superparamagnetic iron oxide nanoparticles (SPIONs). The labeling procedure did not affect tumor cell proliferation and viability. By T2* weighted MRI we were able to visualize single tumor cells in the brain. We developed a fully automated MRI-based quantification program for SPION-labeled cells, and used the program for counting hypointensive

spots in the image data sets from several animal brains, thus we could exclude tumor cell inoculation failures. This model provides valuable biological and therapeutic information on brain metastasis, and improves the success rate when designing preclinical, therapeutic experiments.

Paper IV

Melanoma cells that harbor BRAFV600E mutations and PTEN deletions can successfully be treated with combined therapies, using PLX4032 (BRAFV600E inhibitor) and Temsirolimus (mTOR inhibitor). Combined therapy with these drugs was more effective than single drug treatment. The microarray studies indicated that the combined therapy affects various major downstream functions in melanoma cells, such as cell cycle, cell-death and -survival, cell movement and DNA-replication, - regulation and -repair. Thus, these findings further provide the basis for preclinical therapeutic studies on the animal models for melanoma brain metastatic disease.

6. **Future Perspectives**

As outlined in Paper I, the molecular mechanisms causing brain metastasis have to date only partly been elucidated. However the metastatic models and methodologies that have been described in this thesis, will enable us to better reveal molecular mechanisms responsible for the brain metastatic processes, and to design reproducible and robust preclinical therapy experiments.

Our study (Paper I) shows that there is relatively little overlap between brain metastases genes found in experimental animal models and genes believed to be of importance in the clinic. Using our melanoma brain metastasis model, we have already performed RNA sequencing of tumor cells harvested from different animal organs, to find specific gene signatures responsible for metastatic growth in these organs. Currently, we possess a candidate gene list, which in the coming months will be validated by immunohistochemistry in tissue microarrays from patient brain metastases. In this way, we hope to find genes that can be further knocked in/out, thus finding mechanisms and pathways of importance for the brain metastatic process. Such information may pave the way for defining molecular targets for brain metastases.

Based on the results from this genetic screening, we will be using both molecular inhibition strategies, knock out techniques as well as novel therapy compounds. In this context, the major problem will be to circumvent the blood brain barrier (BBB). Present work in our group (unpublished data) has now shown that the BBB in brain metastasis is very heterogeneous, but leakage of therapeutic substances is usually seen relatively later in tumor development, thus hindering therapeutic efficacy. Thus future work will also focus on opening the BBB for targeted therapeutic delivery.

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