Deregulated epigenetics and cancer stem cells in brain tumours

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To my wonderful parents
As a child, I promised that I would dedicate a book to you. In hindsight, I probably could have chosen an easier one to write…

"*Journey before destination*"
- Brandon Sanderson

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Abstract

Glioblastoma (GBM) is an incurable brain tumour with dismal prognosis as the median survival for afflicted patients is only 8 months. Aberrations of epigenetic processes, which govern gene expression, are involved in cancer, but these epigenetic mechanisms are reversible and can potentially be corrected by treatment. One epigenetic mark, DNA methylation, can also be used for tumour classification. The aim of this thesis was to 1) establish a representative model system of paediatric GBM and use it to identify new epigenetic treatment targets, and 2) profile the epigenetic heterogeneity in adult and paediatric brain tumours and its effect on methylation-based classification.

Patient-derived cancer stem cell (CSC) lines were established in **paper I** from paediatric GBM, and the cells retained the methylation pattern of the originating tumours. The CSC were injected into mice orthotopically in **paper II** and formed GBM tumours similar to the patient tumours. The survival of the injected mice correlated significantly with the survival of the patients, and the model thus reflects the clinical course of the patients. In **paper III**, we performed a CRISPR knockout screen with an epigenetic library and identified several novel genes as essential for the growth of CSC. **Paper IV** demonstrated that multiple methylation subclasses coexist within *adult* GBM, and that the methylation status of the clinically used biomarker *MGMT* also varied. In contrast, the subclasses were stable across space and time in *paediatric* brain tumours in **paper V**.

In conclusion, we showed that intratumour methylation heterogeneity is a feature of adult GBM and should be considered for methylation-based biomarkers and classification. The classification was however homogeneous within paediatric brain tumours, which is promising as it is in clinical use for this patient group. We also established a representative *in vitro* and *in vivo* model system of CSC derived from paediatric GBM. With this model system, we identified candidate genes as tumour drivers and potential therapeutic targets in paediatric GBM.

Keywords

Cancer stem cell, glioblastoma, paediatric, methylation, heterogeneity, CRISPR

Sammanfattning på svenska

Hjärntumörer, dvs. cancer i hjärnan, drabbar 100 barn i Sverige årligen och är den cancertyp som orsakar flest dödsfall hos barn. Vi behöver därför mer kunskap om tumörerna för att komma fram till bättre behandlingar. I **delarbete I-II** etablerade vi därför ett modellsystem av cancerstamceller (CSC) från hjärntumörer hos barn. CSC är speciella cancerceller som tros vara de som orsakar cancern. Vi visade i **delarbete I-II** att vi kan odla CSC på labb på ett sådant sätt att de är väldigt lika den tumören som de kommer ifrån, och cellerna ger även upphov till liknande tumörer när de injiceras i möss. Cellerna utgör således ett representativt modellsystem för hjärntumörer hos barn.

I **delarbete III** använde vi vårt modellsystem av CSC i kombination med gensaxen CRISPR. Vi klippte sönder 1200 gener i CSC med CRISPR och ville hitta de sönderklippta gener som ledde till att cellerna dog eller växte sämre. Vi identifierade flera sådana gener som tidigare inte varit kända som viktiga i hjärntumörer. Vi validerade flera gener och testade en inhibitor (för att stänga av en gen) med lovande resultat, vilket på sikt kan innebära bättre behandlingar.

För att ge patienter rätt behandling krävs en korrekt diagnos av tumören. Diagnos sätts framförallt baserat på hur tumören ser ut i mikroskop, men mycket kan inte ses utan tumörcellernas DNA behöver också undersökas. En förändring i tumörer gäller deras metylering. Metylering innebär att en metylgrupp (CH_3; kol med tre väten) finns på vissa ställen på DNA-molekylen och baserat på metyleringsmönstret kan tumörerna diagnosticeras bättre. I **delarbete IV** studerade vi hjärntumörer hos vuxna och konstaterade att metyleringsmönstret skiljde sig åt på olika ställen inom samma tumör och klassades som olika diagnostiska undergrupper. Eftersom metylering är ett av de verktyg som används för att ställa diagnos på barn med hjärntumörer verifierades i **delarbete V** att samma diagnos gavs på olika ställen inom samma hjärntumör hos barn.

Sammanfattningsvis har denna avhandling 1) utrett hur metyleringsmönstret skiljer sig inom hjärntumörer och hur det påverkar diagnos, 2) tagit fram modellsystem för hjärntumörer hos barn och 3) identifierat viktiga gener för tillväxt av CSC. Behandling skulle kunna riktas mot dessa gener/proteiner och kan på sikt potentiellt leda till att barn med hjärncancer lever längre.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Wenger A**, Larsson S, Danielsson A, Elbæk KJ, Kettunen P, Tisell M, Sabel M, Lannering B, Nordborg C, Schepke E, Carén H. Stem cell cultures derived from pediatric brain tumors accurately model the originating tumors. *Oncotarget. 2017;8(12):18626-39*.

II. Larsson S, **Wenger A**, Dósa S, Sabel M, Kling T, Carén H. Cell line-based xenograft mouse model of paediatric glioma stem cells mirrors the clinical course of the patient. *Carcinogenesis. 2018; 39(10):1304-1309*.

III. **Wenger A**, Karlsson I, Kling T, Carén H. CRISPR-Cas9 knockout screen identifies essential genes for paediatric glioma stem cell growth. *Manuscript*.

IV. **Wenger A**, Ferreyra Vega S, Kling T, Olsson Bontell T, Jakola A.S., Carén H. Intratumor DNA methylation heterogeneity in glioblastoma: implications for DNA methylation-based classification. *Neuro-oncology. 2019;21(5):616-27*.

V. **Wenger A**, Ferreyra Vega S, Schepke E, Löfgren M, Olsson Bontell T, Tisell M, Nilsson D, Kling T, Carén H. DNA methylation alterations across time and space in paediatric brain tumours. *Manuscript.*

Additional publications not part of this thesis:

i. Kling T*, **Wenger A***, Beck S, Carén H. Validation of the MethylationEPIC BeadChip for fresh-frozen and formalin-fixed paraffin-embedded tumours. *Clinical epigenetics. 2017;9:33*. * Shared first author.

ii. **Wenger A**, Larsson S, Carén H. Patient-derived cells modeling pediatric glioma. *Aging. 2017;9(5):1353-4*.

iii. Tüzesi A, Kling T, **Wenger A**, Lunavat T, Jang S, Rydenhag B, Lötvall J, Pollard S.M., Danielsson A, Carén H. Pediatric brain tumor cells release exosomes with a miRNA repertoire that differs from exosomes secreted by normal cells. *Oncotarget. 2017;8(52):90164-90175*.

iv. **Wenger A**, Werlenius K, Hallner A, Thorén F, Farahmand D, Tisell M, Smits A, Rydenhag B, Jakola A.S., Carén H. Determinants for effective ALECSAT immunotherapy treatment on autologous patient-derived glioblastoma stem cells. *Neoplasia. 2018;20(1):25-31*.

v. Li J, Xue Y, **Wenger A**, Sun Y, Wang Z, Zhang C, Zhang Y, Fekete B, Rydenhag B, Jakola A.S., Jiang T, Carén H, Fan X. Individual Assignment of Adult Diffuse Gliomas into the EM/PM Molecular Subtypes Using a TaqMan Low-Density Array. *Clinical Cancer Research. 2019; 25:7068-77*.

vi. Kling T, **Wenger A**, Carén H. DNA methylation-based age estimation in pediatric healthy tissues and brain tumors. *Aging (Albany NY). 2020; 12: 21037-56*.

Content

Abbreviations

ATCC	American Type Culture Collection
ATRT	atypical teratoid rhabdoid tumour
ATRX	α-thalassemia mental retardation X-linked
bp	base pair
Cas	CRISPR associated
Cas9	CRISPR associated protein 9
cfDNA	cell-free DNA
CIMP	CpG island methylator phenotype
CpG	cytosine-phosphate-guanine
CNA	copy-number alteration
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	crisprRNA
CSC	cancer stem cell
dCas9	dead Cas9
DAPI	4',6-diamidino-2-phenylindole
DAXX	death-domain associated protein
DEPMAP	dependency map
DIPG	diffuse intrinsic pontine glioma
DMEM/F-12	dulbecco's modified eagle medium/nutrient mixture F-12
DMP	differentially methylated position
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EdU	5-ethynyl-2-deoxyuridine
EGF	epidermal growth factor
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FFPE	formalin-fixed paraffin-embedded
GBM	glioblastoma
G-CIMP	glioma CpG island methylator phenotype
GFAP	glial fibrillary acidic protein
gRNA	guide RNA
ICE	inference of CRISPR edits
IDH	isocitrate dehydrogenase

indel	insertion-deletion
INFORM	INdividualised therapy FOr Relapsed Malignancies
ITCC	Innovative Therapies for Children with Cancer
MAGeCK	Model-based Analysis of Genome-wide CRISPR Knockouts
MAP2	micro-tubule associated protein 2
MATCH	Molecular Analysis for Therapeutic CHoice
MGMT	O6-methylguanine-DNA methyltransferase
MNP	Molecular Neuropathology
NHEJ	non-homologous end joining
NSC	neural stem cell
OLIG2	oligodendrocyte transcription factor 2
PAM	protospacer adjacent motif
PBS	phosphate-buffered saline
RNA	ribonucleic acid
RNP	ribonucleoprotein
SAM	S-adenosyl methionine
SHH	sonic hedgehog
SOX2	(sex determining region Y)-box 2
TALEN	transcription activator-like effector nuclease
TET	ten-eleven translocation
TMZ	temozolomide
tracrRNA	trans-activating CRISPR RNA
WHO	World Health Organisation
ZFN	zink-finger nucleases

Introduction

Cancer

Cancer is a complex, dynamic and heterogeneous disease with genetic alterations such as mutations and chromosomal copy-number alterations (CNAs), but also epigenetic alterations. Shared traits/capabilities across cancer types have been summarised into eight core hallmarks of cancers, two emerging hallmarks and four enabling characteristics[1-3]. Among the core hallmarks are several capabilities regarding sustained proliferation, resisting apoptosis, evading growth suppressors and the immune system. The characteristics enabling these capabilities include genome instability and nonmutational epigenetic reprogramming (further discussed in *Epigenetics* chapter). These two are also involved in the update of Knudson's two-hit hypothesis stating that two hits, through either mutation or epigenetic silencing, is required to inactivate a tumour suppressor gene and enable tumour initiation[4,5]. Additional hallmarks of cancer have been added since the first edition was published in 2000, highlighting the intricate complexity of cancer and our increased understanding of it.

Childhood cancer

The frequency of cancer increases with age in adults and cancer generally arises due to a lifetime of accumulated mutations and genetic alterations, sometimes linked to environmental risk factors and life style choices (e.g. smoking as risk factor for lung cancer)[6-9]. Children on the other hand have a limited time span to accumulate genetic alterations, and childhood tumours consequently have much fewer mutations and CNAs compared to adult tumours (**Fig. 1**)[10,11]. Certain childhood cancers such as the brain tumour pilocytic astrocytoma have very "silent" genomes with no, or very few, genetic alterations[11]. The mutations found in paediatric tumours are in general also different from adulthood cancer, frequently targeting genes involved in epigenetic and developmental regulation[12-14]. Further, the same cancer type differs substantially when it presents in adults compared to children, e.g. the brain tumour glioblastoma (GBM) and acute myeloid leukaemia[15-17]. The types of cancers also vary between the age groups; cancer in the breast, prostate, lung and colon are the most common in adults while

leukaemia, central nervous system (CNS; the brain and spinal cord) tumours and lymphomas are the most prevalent in children[7]. Some cancer types (e.g. neuroblastoma) are almost unique to childhood and occur at specific age intervals[18,19]. The compiled findings indicate that there are different mechanisms for tumourigenesis in adult versus childhood cancer. Almost 10% of all childhood cancer can be explained by a mutation in a cancer predisposition gene (e.g. *TP53*) in the germ line[11,20,21], but the cause in the remaining cases is largely unknown. The low level of mutations (but frequent in epigenetic pathways) combined with the uniqueness of certain tumour types in childhood suggest a developmental window for paediatric cancer driven by epigenetic deregulation[14,22-24] as further outlined in the *Epigenetics* chapter.

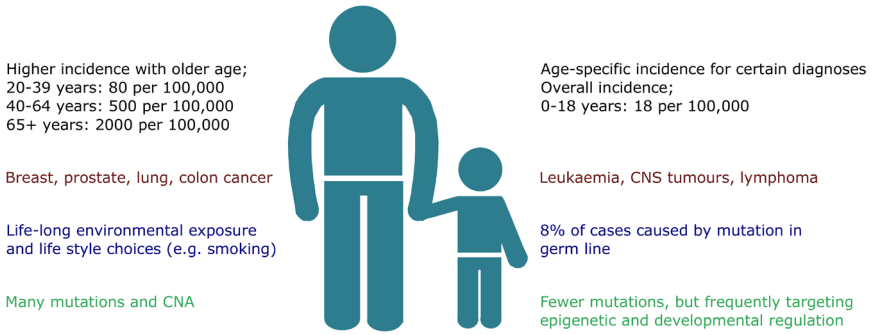

Figure 1. Cancer in adults vs children*. Cancer differs in adults (left) and children (right) regarding incidence, the most common cancer types, cause (e.g. smoking in lung cancer in adults vs germ line mutations in children), genetic copy-number alterations (CNAs) and mutations.*

Paediatric cancer epidemiology

The most common type of childhood cancer is leukemia (28%), followed by CNS tumours (27%), which causes the most cancer-related deaths in children[7,25]. Childhood cancer is fortunately relatively rare (incidence 18 per 100,000; 350 children yearly in Sweden), and survival rates have increased significantly during the last four decades from 58 to 86%[7,26], largely due to the introduction of multimodal treatment (surgery, chemotherapy and radiotherapy). However, the prognosis varies substantially by cancer type, and progress has stalled for high-grade tumours, leaving cancer as the second most common cause of death for children in developed countries[7]. Further, childhood cancer survivors frequently suffer from side effects from the treatment in the form of learning deficits, severe

or disabling chronic health conditions and psychosocial issues[27,28]. Novel therapies specifically targeting the cancer cells are therefore needed to improve survival rate and decrease the severe side effects for survivors of childhood cancer.

CNS tumours

CNS tumours are very diverse with more than 100 diagnoses of varying aggressiveness spanning from benign tumours to lethal[7,29]. The tumours are classified through a combination of histopathology and molecular diagnostics, including mutation status and deoxyribonucleic acid (DNA) methylation[29]. They are also graded according to the CNS World Health Organisation (WHO) scale of 1-4, with 4 denoting the most malignant/aggressive tumours[29]. Some of the most common categories of CNS tumours include gliomas (arising from glial cells), embryonal tumours (childhood tumour medulloblastoma is one of the most common in this category), and meningiomas (arising from the meninges, which is a membrane surrounding the brain and spinal cord)[29,30]. This thesis will mainly focus on the aggressive tumour form GBM in children (paper I-III) and adults (paper IV), whereas paper V includes various childhood CNS tumours e.g. medulloblastoma and ependymoma. The following sections provide an overview of the most common diagnoses and treatments in brain tumours in children and adults, with a focus on GBM and how it differs in children vs adults.

Paediatric brain cancer diagnoses

CNS tumours in children is a heterogeneous group regarding diagnoses, locations, prognosis and it is also worth noting the large age span of this patient group and corresponding biological differences between an infant and a child about to turn 18 years. Consequently, certain tumours are more frequent in older vs younger children, e.g. the incidence of tumours of the pituitary (predominately non-malignant) increases with age in children, whereas medulloblastoma and gliomas decrease with age in this patient group[25]. The most common types of brain cancer in children overall are pilocytic astrocytomas (glioma grade 1), other gliomas, followed by embryonal tumours, of which medulloblastomas are the most frequent[25]. CNS tumours usually arise in the cerebellum (e.g. medulloblastoma and pilocytic astrocytoma), brain stem (e.g. diffuse intrinsic pontine glioma; DIPG) and the pituitary and chraniopharyngeal ducts (chraniopharyngeoma)[25].

Depending on the tumour type and its location, the children present with symptoms such as headache, nausea/vomiting, hydrocephalus, fatigue, seizure, vision impairment, balance issues and delayed development[31].

The differences between adult and childhood cancer exist also within CNS tumours, starting with the overall frequency where CNS tumours constitute approximately 1% of cancers in adults and, as stated above, almost 30% of paediatric cancers[7,25]. The main diagnosis in this thesis, GBM, differ significantly between the age groups in terms of frequency (14% of CNS tumours in adults and 3% in children)[25], amount of mutations and CNAs, as well as the type of mutations. The dismal prognosis is unfortunately similar in all age groups. Adult GBM has more mutations and CNAs, while around 20% of paediatric GBM show minimal number of CNAs[10,11,15]. These differences were recognized in the current 2021 CNS WHO classification where diffuse gliomas are now divided into "*adult-type*" and "*paediatric-type*" gliomas[29]. It should be noted that the diagnosis GBM is not used for paediatric patients as of the WHO 2021 classification as they are now divided into one of the four types of *paediatric-type high-grade gliomas*[29]. They are stratified based on their location and mutation status of histone 3, which is a mutation unique to childhood tumours[14,29,32] (further discussed in *Cancer epigenomics* below). Papers I and II were published prior to the 2021 classification and thus use the old naming convention of GBM, as does the majority of the referenced studies herein. These tumours will therefore be referred to as GBM throughout this thesis. Similarly, tumours referred to as DIPG here are termed *Diffuse midline glioma H3, K27 altered* according to the WHO 2021 classification[29,33].

In addition to the *paediatric-type diffuse high-grade gliomas*, there is also a corresponding *paediatric-type diffuse low-grade glioma* category with expected better prognosis. In contrast to the diffuse and infiltrative growth pattern in these categories, there is also a family termed *circumscribed astrocytic gliomas* containing tumours with well-defined boundaries. This category includes pilocytic astrocytoma, the most common brain tumour in children (15% of cases), which is a slow-growing grade 1 tumour with a 5-year survival of 97%[25].

Medulloblastoma, a tumour arising from remaining embryonic cells in the brain, can occur at any age (including young adulthood in rare cases), but is most common in younger children[25]. It is an aggressive grade 4 tumour with an overall

5-year survival of 65-70%[25,34]. Fifteen years ago, medulloblastoma was subclassified solely based on histological features (e.g. large cell, desmoplastic)[35]. Since then, molecular subgroups of clinical and biological relevance have been identified based on transcriptional[36] and methylation profiling[37-39] and included in the WHO classification[29] currently containing four molecular subgroups; wingless (WNT)-activated, Sonic hedgehog (SHH)-activated and *TP53*-wildtype, SHH-activated and *TP53*-mutant, and non-WNT/non-SHH (commonly referred to as Group 3 and 4 where the driver genes are unknown). The SHH-activated medulloblastoma have a mutation in a germline predisposition gene (*TP53*, *PTCH1* etcetera) in around 20% of cases[11]. It should be mentioned that further subtypes within the four subgroups have also been identified through methylation profiling, e.g. eight subtypes within the non-WNT/non-SHH category[40].

Ependymoma is a type of glioma arising from ependymal cells lining the ventricles. The previous WHO classification from 2016 included 7 subgroups[33], mainly defined through histology, but they showed poor correlation with tumour grade and patient outcome[41]. The classification has therefore undergone a major transformation in terms of clinically and biologically relevant subgroups identified e.g. through DNA methylation profiling[42,43]. The overall 5-year survival for ependymoma is almost 80%[25], but identified subtypes range between 50 and 95%[44]. In the current WHO 2021 classification, ependymoma are divided based on an integrated assessment of location (e.g. posterior fossa and spinal), molecular markers (*MYCN* amplification, *ZFTA* or *YAP1* fusion genes) and histological features into 10 subgroups (grade 1-3)[29,43].

Paediatric brain cancer treatment and side effects

The treatment is largely dependent on the tumour type, its location and the age of the patient. Very young children are for instance not treated with radiotherapy to avoid damage to the developing brain. For low-grade tumours such as pilocytic astrocytoma, complete resection of the tumour may be the only treatment needed. Follow-up MRI scans monitor if the tumour grows and additional treatment in the form of chemotherapy and radiation (for older children) or chemotherapy alone (younger children) is then added[45]. Pilocytic astrocytoma has a 5-year survival of 97% for children, which is in stark contrast to GBM with a 5-year survival of 16% in children[25]. There is no clear consensus on the optimal treatment for paediatric GBM, but the standard treatment regimen for adults is commonly used. It consists

of gross total resection (which may be difficult to achieve depending on the tumour location and the infiltrative nature of GBM), radiotherapy and chemotherapy (temozolomide; TMZ)[46,47]. Additional chemotherapies have also been added on top of this treatment in the adjuvant setting; e.g. bevacizumab on its own[48], or together with irinotecan[49] or lomustine[50].

Chemotherapy such as TMZ treatment cause toxic side effects including nausea/vomiting, haematologic toxicity (e.g. thrombocytopenia, lymphopenia), and neuropathy (nerve damage), which can manifest as numbness, pain, tingling and balance issues[47,49,51]. As survival for children with brain tumours have improved (5-year survival 76%[25]), the long-term side effects from treatment have come into focus. Childhood brain tumour survivors have subsequently been shown to have deficits in memory, processing speed and attention span[52,53]. The deficits in learning also lead to a decrease in intelligence, lowered academic achievements and higher risk of unemployment[54-56]. Younger age at diagnosis is also associated with more severe side effects[52,57]. Further, the children also have social deficits[58] and higher incidence of depression, anxiety and behavioral issues compared to their peers[59]. Radiotherapy is considered to have the highest risk of causing neurological deficits[57], but children treated with surgery alone (without chemo/radiotherapy) also have deficits in intelligence and adaptive behavior[60].

Adult brain cancer diagnoses and treatment

The most common malignant brain tumour in adults is GBM (a diffuse glioma grade 4), representing almost half of the malignant tumours and 14% of all CNS tumours[25], and, as described above, the disease differs markedly between children and adults. Among the non-malignant tumours, almost half are meningioma and it comprises almost 40% of all brain tumours[25]. GBM commonly arise in the cerebral hemispheres in a single lobe (particularly the frontal or temporal lobe) or multiple lobes, and patients usually present with symptoms such as headache, neurological deficits, cognitive disorders, seizures and nausea/vomiting[61]. Median age at diagnosis is 65 years[25] and the risk of GBM increases with older age[62].

The *adult-type diffuse gliomas* are diagnosed mainly based on *isocitrate dehydrogenase 1 and 2* (*IDH1, IDH2*) mutations, which are most prevalent in lower-grade gliomas, whereas GBM is IDH-wildtype[63]. The IDH enzymes are crucial for metabolic pathways including Kreb's cycle, and *IDH* mutation leads to

neomorphic activity (novel molecular function) and subsequent alterations in the cellular metabolism and epigenetic pattern (see description on CIMP in *Cancer epigenomics*)[64]. *IDH* mutated glioma patients are generally younger and have a better prognosis compared to *IDH*-wildtype gliomas (GBM), which has a 5-year survival of less than 5%[25].

Standard treatment for adult GBM is based on maximal safe tumour resection, followed by concomitant and adjuvant chemotherapy (TMZ) and radiotherapy resulting in a median survival of 15 months[46]. Patients with a methylated *O6-methylguanine-DNA methyltransferase* (*MGMT*) gene promoter (further described in *Methylation-based biomarkers*) has been shown to respond better to TMZ treatment[65]. The standard treatment of GBM has unfortunately not advanced since the introduction of TMZ in 2005 despite intense research efforts and several clinical trials. The exception is a clinical study with tumour-treating electrical fields, which in combination with standard treatment prolonged median overall survival with almost 5 months[66].

Tumour heterogeneity and cancer stem cells

Cancer is a heterogeneous disease with large differences between cancer types, but also within tumours. The cancer cells within a tumour differ in terms of mutations, CNAs, gene expression, epigenetic alterations, differentiation state, signals from the tumour microenvironment, and response to therapy[67-70]. Intratumour heterogeneity in adult GBM is well-described on genetic and transcriptional levels[71-73], but how the epigenetic machinery differs within tumours is less investigated, which paper IV sought to address. Regional spatial heterogeneity in paediatric GBM and DIPG has been shown regarding histopathology (e.g. low-grade and high-grade components), diverging subclones, transcriptome, CNAs and mutations[74-76].

There are two proposed models for tumour heterogeneity in cancer; the stochastic model and the cancer stem cell (CSC) model (**Fig 2**). The stochastic model (also called clonal evolution) is based on a normal cell acquiring neoplastic genetic/epigenetic alterations that provide a growth advantage. Over time, this cell acquires further alterations due to genetic instability, and thus generates subpopulations/tumour clones[77]. The clone with the best growth advantage will dominate the population until new alterations leading to additional growth

advantages take over. In contrast, the CSC model (also called hierarchical model) states that only a small subpopulation of cells, the CSC, are capable of self-renewal and tumourigenesis and they give rise to differentiated tumour cells in a hierarchical manner[78,79]. A CSC has been defined as "*a cell within a tumour that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumour*"[80]. It means that CSC should be capable of self-renewal, differentiation, express stem cell markers, and form tumours upon transplantation *in vivo*.

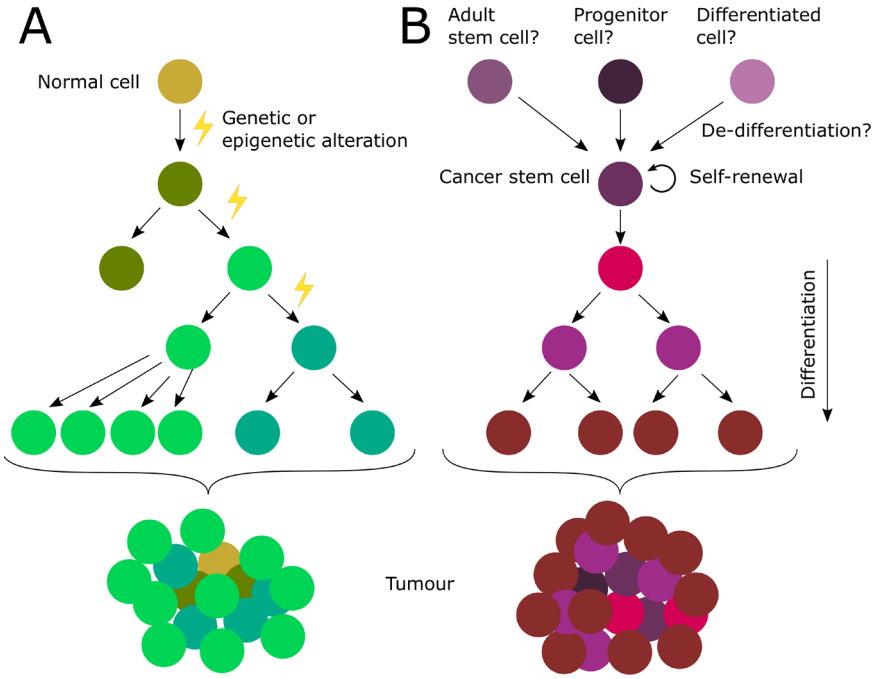

Figure 2. Stochastic and cancer stem cell model. *A) According to the stochastic model, any non-cancer cell can acquire a growth advantage and neoplastic properties through genetic/epigenetic alterations. The tumour cell generates subclones and they in turn gain further advantageous alterations and expand until another clone takes over the population, thus resulting in a heterogeneous tumour. B) The cancer stem cell (CSC) model states that only a small subpopulation of cells, the CSC, can initiate a tumour. The CSC are capable of self-renewal and differentiation, and give rise to a heterogeneous tumour with cells at varying differentiation stages.*

The existence of CSC has been demonstrated in several tumours, including GBM[81-84], where they are also referred to as glioma stem cells. They can also be called tumour initiating cells, cancer progenitor cells or cancer stem-like cells.

Regardless of the name, these cells are resistant to chemo and radiation therapy and are therefore believed to be a driver in tumour growth and tumour recurrence[85-87]. Suggested factors contributing to their resistance are their plasticity, slow proliferation rate and ability to enter a quiescent state, thus escaping therapies targeting dividing cells or specific cell states[88]. The origin of CSC is unclear and speculations place the origin as de-differentiated mature cells, progenitor cells or tissue-specific stem cells[89]. CSC from the brain has been suggested to originate from neural stem cells (NSC) in the subventricular zone[90].

Cancer model systems

Pre-clinical model systems of cancer are vital to elucidate the mechanisms underlying tumourigenesis, and for developing and evaluating novel treatments. One of the most common model systems is *in vitro* cell cultures derived from tumours, going back to 1951 when the first cancer cell line (HeLa) was established from a patient with ovarian cancer[91]. Cells with the ability to proliferate indefinitely have subsequently been derived from most tumour types. These so-called traditional cell lines are available to researchers worldwide through cell repositories (e.g. American Type Culture Collection; ATCC). However, traditional cell lines adapt to an artificial environment in cell medium containing serum and accumulate genetic alterations in culture over decades of cell culture up to a point where they do not resemble the tumour of origin[92-94]. Traditional tumour cell lines have in fact been shown to be more similar to each other, regardless of tumour type, than to the tumours they were derived from and supposedly model[92,95]. Consequently, their validity as a representative model system is questioned. Further, the lack of clinical data (e.g. survival) precludes correlations between results in the cell lines and clinical parameters.

In contrast to traditional cell lines, there are serum-free patient-derived primary cell lines, where the cells are used in relatively early passages. They are more similar to the patient tumour than the traditional serum-cultured cell lines, and as such constitute a representative model system[96]. In recent years, it has therefore been a transition towards primary cells and “personalized medicine” to increase the chances that results from cell-based experiments are transferable to the cancer *in situ* and hopefully to the benefit of the patients. The following sections present an overview of *in vitro* and *in vivo* mouse model systems for paediatric and adult GBM.

In vitro glioblastoma model systems

The traditional adult and paediatric GBM cell lines (e.g. U87, U251, KNS-42) are cultured with serum, which induces astrocytic differentiation of GBM stem cells and depletion of tumour-initiating cells (CSC)[96-99]. In contrast, serum-free adherent cultures of adult glioma stem cells (CSC) have successfully been established by multiple groups and the cells accurately represent the originating tumours[96,100-102]. Adult GBM cell lines are therefore common whereas childhood GBM cell lines are rare[97], and we established the first serum-free paediatric GBM culture in 2017 (paper I)[103]. Subsequent cell cultures are scarce, but Brabetz et al. employed a similar protocol as ours to establish serum-free paediatric GBM cell lines[104]. Another group used a different approach where they first transplanted freshly dissociated tumour cells into mice and then derived serum-free GBM cultures from the xenograft tumours[105]. Regarding DIPG, another paediatric high-grade glioma, serum-free cultures have been established as tumour spheres[106].

In vivo glioblastoma model systems

Animal models are another important pre-clinical model system as they allow for interaction of the tumour microenvironment, signaling from other cell types and overall a systemic model more representative of cancer compared to *in vitro* culture in a plastic dish. Mouse models of adult GBM mainly consist of subcutaneous or orthotopic transplantation of tumour tissue, freshly dissociated tumour cells or cultured cells[107]. Xenograft models of serum-free adult GBM cells initiate tumours with invasive growth patterns characteristic of human GBM whereas transplanted traditional cell lines (e.g. U87) lack such features[99,100,102,108].

Paediatric *in vivo* models are less common, but we also observed an invasive growth pattern and similarity to the patient tumour upon orthotopic transplantation of our paediatric GBM cells[109] (paper II). Other paediatric CNS tumour *in vivo* models are generally based on implantation of freshly dissociated tumour cells (never cultured), but also report that the xenograft tumours are histologically similar to the patient tumour[104,105].

Epigenetics

The term epigenetics was coined by Conrad H. Waddington already in 1942 referring to cell differentiation and development that was not related to changes in the genotype[110]. Epigenetics has later been defined as the "*molecular factors and processes around DNA that are mitotically stable and regulate genome activity independent of DNA sequence*"[111]. The DNA sequence is the same in all cells within an individual, thus epigenetics regulates which genes that are expressed in various cell types leading to the diverse cell phenotypes in the body. It is therefore logical that epigenetics plays a fundamental role during embryogenesis, development, X-chromosome inactivation, aging, cell growth, cell differentiation etcetera, and aberrations in the epigenetic machinery have been linked to several diseases including cancer[112] (described further in *Cancer epigenomics*).

The epigenetic machinery consists of three main mechanisms; histone modifications, non-coding ribonucleic acid (RNA) and DNA methylation (**Fig. 3**). Histones are the proteins that fold and package the DNA into chromatin. Duplicates of the four histone proteins H3, H4, H2A and H2B form an octamer, around which a 147 base pair (bp) DNA segment is wrapped to form a nucleosome[113]. Histone modifications, including methylation, phosphorylation and acetylation, occur on the histone tails (a protrusion from the core histone), and the modifications alter the chromatin structure, which can affect gene expression[114]. Non-coding RNA is, as the name implies, not translated into a protein, but are nonetheless able to regulate gene expression through chromatin modulation and binding[115,116].

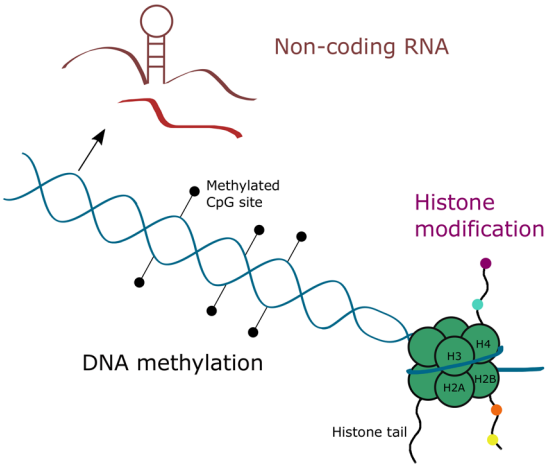

Figure 3. The epigenetic machinery. *The three main epigenetic mechanisms are non-coding RNA, DNA methylation and histone modifications. DNA methylation consists of the addition of a methyl group to a cytosine in a CpG site (cytosine followed by guanine). Histones are the proteins which fold and package the DNA. Histone modifications (illustrated as coloured circles) occur on the histone tails.*

DNA methylation

The third epigenetic mechanism, and the most studied, is DNA methylation, which was first described as early as the 1940s[117]. DNA methylation consists of the addition of a methyl group (CH_3) to the carbon on the fifth position in a cytosine, which almost exclusively occurs on a so-called CpG site (cytosine-phosphate-guanine; i.e. a cytosine DNA base followed by a guanine base)[118]. The reaction is catalysed by a family of DNA methyltransferases (DNMTs). They methylate the cytosine in the CpG site by transferring a methyl group from the methylation donor S-adenosyl methionine (SAM; **Fig. 4**)[119,120]. DNMT1 maintains already established methylation patterns whereas DNMT3 carries out *de novo* methylation[121,122]. Due to their role, DNMTs are referred to as methylation writers, and they are complemented by methylation erasers and readers. The removal of methylation can be either passive or active, through several proposed mechanisms, one of which is catalysed by the methylation erasers ten-eleven translocation (TET) enzymes[123-125]. The methylation readers recognise the methylation, bind to it and mediate the effect through additional factors to repress transcription[126,127]. Mutation of the methylation reader MeCP2 has been shown to cause Rett syndrome (severe mental retardation)[128] whereas knockout of the methylation writer DNMT1 leads to the termination of embryos[129], highlighting the importance of these enzymes.

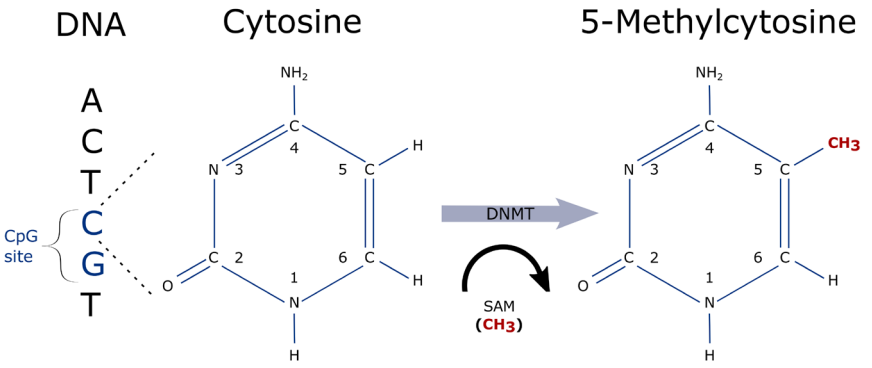

Figure 4. CpG methylation*. DNA methylation mainly occurs at CpG sites (cytosine followed by guanine). A methyl group (carbon with three hydrogen; CH_3) is transferred from S-adenosyl methionine (SAM) by DNA methyltransferases (DNMT) to the carbon (C) at the fifth position of cytosine to form methylated cytosine (5-methylcytosine).*

The human genome contains almost 30 million CpG sites and the majority are methylated in human somatic cells[130]. A region with many CpG sites in close proximity is termed a CpG island[131]. The regions next to the CpG islands has been defined as shores (<2 kb away), followed by shelves (2-4 kb away) and then the Open Sea, with the frequency of CpG sites decreasing with distance from the CpG island[132] (**Fig. 5A**). CpG islands are the most studied as they are frequently located in gene promoter regions, with more than half of mammalian gene promoters being associated with a CpG island[133]. DNA methylation has been shown to regulate gene expression through the methylation state of the promoter region; an unmethylated promoter allows for gene expression whereas a hypermethylated promoter silences gene transcription[130,134] (**Fig. 5B-C**). This regulation of gene expression is critical e.g. in embryogenesis, imprinting, development and cell differentiation[120,135,136].

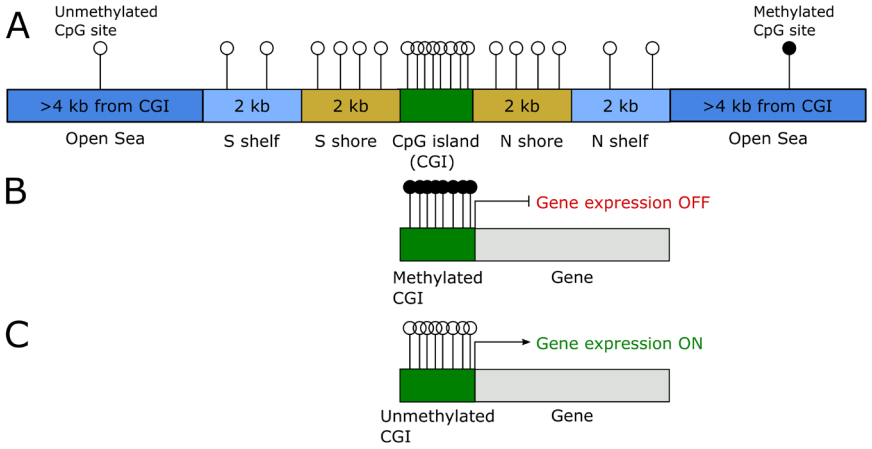

Figure 5. DNA methylation and gene expression*. A) CpG sites are most frequent at CpG islands (CGI) and more sparse further away in the shores, shelves and the OpenSea. B) DNA methylation in the CGI can regulate gene expression where a methylated CGI represses gene expression and C) an unmethylated CGI allows for gene expression.*

Cancer epigenomics

The 2022 updated revision of the *Hallmarks of Cancer* included deregulated epigenetics as an enabling characteristic of cancer[3]. The epigenetic pattern is widely altered in cancer where several epigenetic factors work together in synchrony to modify the packaging of the chromatin[137-139]. The chromatin structure alters the accessibility of the DNA, which in turn modulates the gene expression[140-

[142]. Epigenetic alterations are however reversible and can, in theory, be treated to revert or differentiate cancer cells into a non-neoplastic cell state. Several "epigenetic drugs" have been approved by the US Food and Drug Administration (FDA), including the DNMT inhibitors 5-azacytidine and decitabine, and the histone deacetylase inhibitors vorinostat and valproic acid. Several clinical trials are currently ongoing to evaluate epigenetic drugs in paediatric brain tumours[143]. The following sections will focus on the most common histone mutations and methylation aberrations in cancer, particularly in brain tumours, and its utility for tumour classification.

Global hypomethylation, mostly occurring in repetitive regions, is a universal feature of solid cancers, leading to chromosomal instability and increased mutation frequency[144-146]. The mechanisms that induce these changes are unknown, but studies suggest that the alterations occur early in tumour development[147] and are present in normal tissue prior to tumourigenesis[148-150]. There are also specific alterations promoting tumourigenesis through hypomethylation (i.e. activation) of oncogenes and hypermethylation (silencing) of tumour suppressor genes[134,151]. Epigenetic silencing has been proposed to act as one of the hits[5] in Knudson's two-hit hypothesis[4]. A genome-wide hypermethylation of CpG islands was dubbed *the CpG island methylator phenotype* (CIMP), when it was first described in colorectal cancer more than twenty years ago[152]. Ten years later (2010), it was shown in a subset of adult gliomas of low-grade (glioma CIMP; G-CIMP), with a very strong association to *IDH1* mutation, the molecular marker used for stratifying adult glioma diagnoses[153,154].

Paediatric tumours, including CNS tumours, have fewer mutations than adult tumours, suggesting a particular importance of deregulated epigenetics in childhood cancer[10,11]. The *IDH1* mutations commonly occurring in younger adults with lower-grade gliomas are rarely observed in paediatric glioma[16]. However, driver mutations in histone *H3.3* (*H3F3A* or *HIST1H3B/*C) and the chromatin remodelling genes *ATRX (α-thalassemia mental retardation X-linked*) and *DAXX* (*death-domain associated protein*) have been identified in almost half of paediatric GBM and up to 80% of DIPG[14,32]. The mutations cause an amino acid substitution (K27M or G34R) on the histone tail, which lead to widespread changes in gene expression and epigenetic aberrations, e.g. through DNA methylation, specific for the location and mutation[14,16,155,156]. The H3 mutation state (in genes *H3F3A* or *HIST1H3B/C*) and type of mutation (K27 or G34) has been

incorporated in the current WHO 2021 classification of *paediatric-type diffuse high-grade gliomas*[29] and can be identified through methylation-based tumour classifiers. Interestingly, the histone mutations are specific to children and young adults, mainly within GBM and DIPG[14], but has less frequently also been observed in low-grade gliomas[157,158] . It suggests that the defect chromatin pattern, induced by the histone mutation, is a driver of tumourigenesis for this age group.

Methylation-based tumour classifiers

The characteristic methylation pattern, reflecting the cell of origin and cancer alterations[159], has proven useful in tumour classification and subclassification[16,155,160-162]. Unlike the standard histopathological evaluations, which suffer from inter- and intra-observer variability[41,163], methylation-based profiling provides an unbiased diagnosis that can be used as a complement to histopathology. Methylation profiling has also identified novel biologically and clinically relevant tumour subgroups where the medulloblastoma and ependymoma subgroups (as described above)[42,164,165] are included in the latest edition of the CNS WHO classification[29,33]. In light of this progress, multiple methylation-based tumour classifiers have been developed over the last few years. One classifier diagnosed metastases of unknown primary origin by matching its methylation pattern to the cancer type with most similar profile[166]. Several classifiers specific for CNS tumour have also been developed by multiple groups[155,160,167]. The most widely used is the Molecular Neuropathology (MNP) classifier by Capper et al.[155], which is also used clinically for paediatric brain tumours in several countries, including Sweden[168-170].

Methylation-based biomarkers

In addition to methylation classifiers, there are also methylation-based biomarkers, where *MGMT* is the most well-known and in clinical use for treatment decision in elderly GBM patients[171]. *MGMT* codes for a DNA repair enzyme that counteracts the alkylating TMZ treatment[172]. Patients with an active *MGMT* promoter (unmethylated) therefore have no benefit of TMZ treatment, and are given radiation only as treatment, while patients with a silenced (methylated) *MGMT* promoter receive TMZ in addition to radiotherapy[65,171,173].

Additional methylation-based biomarkers, not in clinical use, is G-CIMP where adult glioma patients with G-CIMP have a better prognosis compared to patients without G-CIMP[153]. Another suggested biomarker is age-estimators based on the methylation-profile[174-176]. Several such "epigenetic clocks" have been developed and age acceleration (higher methylation age than chronological age) has been shown to occur in cancer and to vary across GBM subtypes[174,175]. We did a study on paediatric brain tumours and found that the age acceleration was subtype-specific for ependymoma, medulloblastoma, glioma and atypical teratoid rhabdoid tumours (ATRT)[177]. For three of these tumour types, the subtype with highest acceleration corresponded to the worst prognosis suggesting the potential use of methylation age as a prognostic biomarker in childhood CNS tumours.

Genome engineering

Genome engineering allows for specific alterations in the genome, and the first method, zink-finger nucleases (ZFN), was developed in 1996[178] and followed by transcription activator-like effector (TALE) nucleases (TALEN) in 2010[179]. These methods are time-consuming as protein engineering is needed to alter the DNA-binding modules and target another genomic locus. The newest genome engineering technique is called clustered regularly interspaced short palindromic repeats (CRISPR). It is originally part of the bacterial immune system, but was repurposed 2012 for genome engineering in mammalian cells[180-182]. It was rapidly applied in basic life science, clinical trials to cure heritable diseases, agriculture to create resistant crops, and research on COVID-19 detection[183-187]. Only eight years after their landmark paper[180], Emmanuelle Charpentier and Jennifer Doudna were awarded the Nobel Prize 2020 for their development of CRISPR as a gene-editing tool.

CRISPR-Cas9 mechanism of action

As mentioned, CRISPR is part of the adaptive immune system in many bacteria and works by cleaving (destroying) foreign genetic elements (bacteriophages, plasmids)[188-190]. The CRISPR locus consists of a CRISPR array, CRISPR associated (Cas) genes and trans-activating CRISPR RNA (tracrRNA)[191,192]. The CRISPR array contains repetitive sequences interspaced by short non-repetitive sequences called spacers, which is a piece of DNA from a past intruder (e.g. bacteriophages) and DNA from new intruders is also incorporated into the CRISPR array[188,193]. The incorporated DNA is next to a protospacer adjacent motif (PAM) site (NGG) in the intruder's genome[194,195]. The spacers are transcribed into CRISPR RNA (crRNA) and anneals to the tracrRNA and this cr:tracrRNA duplex forms a complex with Cas9 (CRISPR associated protein 9) and guides it to cleave the foreign DNA (spacer) with a double strand break occurring 3 bp from the PAM site[180,196].

The CRISPR system has subsequently been modified for editing in mammalian genomes[180-182]. A guide RNA (gRNA) including 20 nucleotides (corresponding to crRNA) is designed to be complementary to the targeted DNA sequence (the "spacer") in the genome, making it easy to alter the targeting site. This sequence must however be directly adjacent to a PAM site (NGG for Cas9). The gRNA

(crRNA:tracrRNA) forms a complex with the nuclease Cas9, and induces a double strand break at the DNA sequence complementary to the gRNA[180]. The DNA break is repaired by the cell mainly through error-prone non-homologous end joining (NHEJ), which frequently creates gene-disrupting mutations in the form of small insertions/deletions (indel) leading to loss of protein (gene knockout; **Fig. 6**)[197-199]. CRISPR can also be used for epigenome editing where dead Cas9 (dCas9; inactive Cas9 mutant) forms a DNA guiding system with a gRNA[200]. dCas9 is fused to various effector domains (*KRAB*, *DNMT3a* etc.) for e.g. transcriptional regulation, DNA methylation, demethylation and histone residue modification at the target location[200,201].

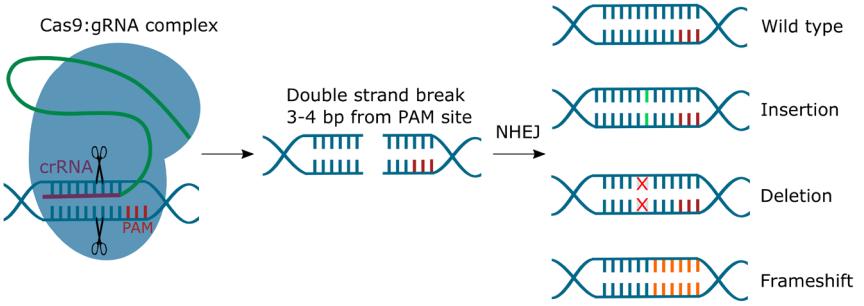

Figure 6. CRISPR-Cas9 knockout. *The Cas9 nuclease and gRNA forms a complex and binds to the DNA that is complementary to the 20 nucleotide long crRNA portion of the gRNA. Note that the crRNA is next to a protospacer adjacent motif (PAM site; NGG for Cas9). The Cas9 nuclease induces a double strand break between the 3rd and 4th bp from the PAM site. The DNA break is repaired by the error-prone repair mechanism non-homologous end joining (NHEJ), which can repair the DNA correctly, but frequently creates small insertions/deletions or frameshifts which can lead to gene knockout.*

CRISPR editing efficiency and off-target effects

The efficiency of the CRISPR edit is affected by the form of the CRISPR components (plasmid, mRNA, protein), method of transfection (lipofection, nucleofection/electroporation, lentivirus), the sequence of the gRNA and the fidelity of the nuclease[202-204]. Delivering the Cas9:gRNA complex as a formed ribonucleoprotein (RNP) have proven to result in less off-target effects (described below) and be more efficient than plasmids, mRNA and lentivirus, with indel formation in 80-90% of the transfected cells[204,205].

DNA cleavage, and subsequent indel formation, at other genomic loci than the intended target is referred to as off-target effects[206]. They can occur at locations with similar DNA sequence as the intended target with up to five mismatches, or with alternative PAM sites (NAG instead of NGG)[207-209]. Off-target effects are therefore a concern for CRISPR-based genome editing, particularly for *in vivo* therapeutic applications[186].

CRISPR in cancer research

CRISPR enables research on all hallmarks of cancer[210] and a wide range of CRISPR-based methods, editing the genome and epigenome, has therefore been applied in cancer research[187,200,201]. This section will mainly describe CRISPR-based gene knockout in cancer, with a particular focus on GBM. One of the most common uses of CRISPR is to identify genetic dependencies for cancer cells (i.e. genes essential for cancer cell growth/fitness), which has mostly been done by pooled genome-wide knockout screens with lentivirus transductions[211-214].

Genome-wide pan-cancer CRISPR knockout screens have been performed in more than one thousand cancer cell lines, mainly through the cancer dependency map (DEPMAP)[214-216], and project Score[213,217]. These massive screens have identified dependency genes in separate tumour types and pan-cancer. However, the screens were performed on traditional cell lines cultured in serum. Screens on patient-derived serum-free adult GBM cultures[100] have been done and they detected tumour drivers outside the networks commonly altered in GBM[218], and identified stemness regulators as essential[219]. Very few knockout screens have been performed on paediatric cell lines, which is needed given the molecular differences between childhood and adult cancer[10,11]. The exception is paediatric DEPMAP[216] with 82 paediatric cell lines, but all were traditional lines and none of them were derived from GBM.

Aims

The overall goal of this thesis was to develop representative model systems of paediatric GBM, and to determine the effect of intratumour epigenetic heterogeneity in adult and paediatric brain tumours on methylation-based classifiers. An accurate diagnosis and subtyping of the tumour is essential for providing an appropriate treatment regimen, and we therefore aimed to investigate how heterogeneity within tumours affects DNA methylation-based diagnostics, which is used clinically for all childhood brain tumours and challenging cases of adult brain tumours. Even with a correct diagnosis and treatment, patients with high-grade brain tumours such as GBM share a very poor prognosis in all age groups. Treatment failure is partly caused by CSC, which are resistant to chemotherapy and radiation and of particular interest to study. However, remarkable molecular differences exist in adult vs childhood GBM, necessitating dedicated model systems and studies on each age group. We therefore also aimed to establish an accurate model system of paediatric GBM to generate knowledge on CSC and tumour progression and identify novel epigenetic tumour drivers and targets for treatment. This could lead to more specific treatments that prolongs survival and decreases side effects for children with GBM.

The specific aims were to:

Paper I&II: Establish a representative patient-derived *in vitro* (paper I) and *in vivo* (paper II) CSC model system for paediatric GBM to enable functional studies

Paper III: Identify epigenetic treatment targets in the CSC derived from paediatric GBM

Paper IV&V: Profile the methylation intratumour heterogeneity in adult (paper IV) and paediatric (paper V) brain tumours, and evaluate implications for DNA methylation-based classification

Materials and methods

The main methods used in this thesis are described below. The reader is referred to the method sections in paper I-V for detailed descriptions on additional techniques.

Patients and samples (paper I-V)

Patient inclusion and sample collection was approved by the Regional Ethical Review Board in Gothenburg (Dnr. 604-12) and carried out in accordance with the relevant regulations and guidelines. Tumour tissue was collected from patients undergoing CNS tumour resection at Sahlgrenska University Hospital after signed informed consent from the patients, or from the parents of paediatric patients. For paper IV and V, we collected spatially separated samples from each tumour and for paper I, we received tissue from a single location. Imprints were made of all tumour samples and the tissue was frozen in liquid nitrogen, for subsequent low-temperature storage and extraction of DNA/RNA, or brought to the lab in PBS (Phosphate-Buffered Saline) for cell isolation and subsequent cell culture (see below). For paper V, we also included patients with paired primary and relapse tumours from a Swedish national methylation study (Dnr: 604-12, T1162-16). The tumour tissue for these samples were received as formalin-fixed paraffin-embedded (FFPE).

Cell isolation and cell culture (paper I-III)

The tumour tissue was mechanically and enzymatically dissociated into single cells, which were cultured adherently on laminin-coated plastics in a serum-free NSC media (containing for example N2 and B27), thus promoting stem cell features, supplemented with epidermal growth factor (EGF), as previously described[100]. EGF is added to the media as studies have shown it necessary for stem cell maintenance and self-renewal[220]. Seven cell lines from paediatric high-grade gliomas were established and used in this thesis. Six of the cell lines are described in paper I, but updated nomenclature as stated in parenthesis below, is used in this thesis; BPC-A7 (GU-pBT-7), BPC-B0 (GU-pBT-10), BPC-B5 (GU-

pBT-15), BPC-B9 (GU-pBT-19), BPC-C3 (GU-pBT-23) and BPC-C8 (GU-pBT-28). The seventh cell line, GU-pBT-58, was established, as described above after the publication of paper I from a 3-year old girl with DIPG.

The cells were cultured in a humidified incubator at 37°C and 7% CO_2. Media was changed every 3-4 days and the cells were passaged with Accutase typically once a week with a split ratio of 1:2-1:7 depending on the cell line. All cell lines were regularly tested for mycoplasma contamination (Eurofins Genomics, Ebersberg, Germany), and the patient identity of the samples was verified with short tandem repeats profiling (IdentiCell, Aarhus University Hospital, Denmark). The established cell lines could be cultured adherently for at least 30 passages without major alterations (see paper I).

As non-cancer control cells in paper III, we used two foetal NSC lines, FT-3465 and FT-3477, derived according to previously established protocols[221,222]. The NSC lines were cultured under the same conditions as the CSC described above, except that fibroblast growth factor (FGF) was also added to the media as it has been shown necessary, in combination with EGF, to isolate NSC[220,223].

CRISPR (paper III)

We performed a CRISPR knockout screen to identify genes essential for CSC growth in paper III. CSC were lentivirally transduced and selected for stable Cas9 expression with blasticidin. A lentiviral CRISPR-Cas9 knockout screen with a chromatin remodelling/epigenetic library containing 1 200 genes[203] and four gRNA/gene was thereafter performed in duplicates. Model-based Analysis of Genome-wide CRISPR Knockouts (MAGeCK)[224] was performed comparing the read counts for each gRNA at the end-point (6 weeks) to the input library, and ranking the genes based on the log fold change and the number of concordant gRNA. The transductions and MAGeCK analysis were performed at SciLife CRISPR Functional Genomics (Stockholm, Sweden). False-positive hits were excluded by removing gRNA with multiple matches in the genome or located in regions where the CSC had CNAs[225,226], and based on lack of gene expression in RNA-sequencing data of the unedited CSC. Hit calling was based on top 250 ranking and three concordant gRNA (out of the four) in both duplicates.

For validation of the CRISPR screen, we used nucleofection (4D-Nucleofector™, Lonza, Basel, Switzerland) to introduce the CRISPR components into the cells as RNP complexes of the Cas9 and gRNA (duplex of crRNA:tracrRNA; IDT, Coralville, USA)[204,205]. After knockout, the cells were analysed for cell confluence in a live-analysis system, cultured for one month, and counted at each split. DNA cleavage at the intended target was verified by ICE (Inference of CRISPR Edits)[227] analysis approximately 10 days after knockout. To evaluate potential off-target effects we also performed ICE analysis on two of the top predicted off-target sites for the gRNA.

In vivo experiments in mice (paper I-II)

The mouse experiments were approved by the animal ethics committee in Gothenburg (Dnr. 10-2015) and conducted according to guidelines from the Swedish National Board for Laboratory Animals. Briefly, 100 000 cells were injected into the frontal cortex of immunocompromised CIEA-NOG mice, as previously described[100]. The mice were monitored and weighed weekly, and were euthanized upon neurological symptoms and/or a 20% weight loss. The mouse brain was removed, fixated and embedded in paraffin prior to sectioning and histological analysis.

In vivo experiments in zebrafish (paper I)

Zebrafish experiments included 1) injection of RFP-labelled cells into the ventricles of zebrafish embryos and visualisation of tumour growth with confocal imaging, and 2) injection of unlabelled cells intranasally into adult zebrafish to create a survival-curve (Dnr. 6-2015; animal ethics committee in Gothenburg).

Histology (paper I, II, IV, V)

Fresh-frozen tumour tissue from mouse brains (paper I and II) and spatially separated regions of the tumours from paper IV were fixated in paraformaldehyde/formaldehyde at 4°C overnight, embedded in paraffin and cut into 5 µm sections prior to de-paraffinisation. These sections, as well as imprints of fresh tumour tissue (paper I and V), were counterstained with Mayer´s

hematoxylin and eosin. The paired temporal tumour samples in paper V had been FFPE-processed by the regional pathology departments. Sections immediately prior to and after the sections used for DNA extraction (and subsequently DNA methylation analysis) were used for histology. Tumour cell content was thereafter evaluated for all samples described above by a specialist in clinical neuropathology.

Immunohistochemistry (paper I-II)

Immunohistochemistry was performed in paper I and II on sections of xenotransplanted mouse brains using a human-specific antibody against NESTIN to allow for detection of the injected CSC and its progeny, and the proliferation marker Ki67. The stainings were performed with a Vecta Stain Kit (Vector laboratories, Burlingame, USA).

Immunocytochemistry (paper I and III)

Analyses of *in vitro* cultured cells included 5-ethynyl-2-deoxyuridine (EdU) incorporation, to measure proliferation, and immunocytochemistry. EdU was added to the cells 24 hours prior to fixation with 4% paraformaldehyde. For immunocytochemistry, the cells were first permeabilised with PBS-Triton (0.1%) to enable the antibodies to enter through the cell membrane, then blocked with goat serum to prevent unspecific binding, and subsequently incubated with the primary antibody binding to the protein of interest. Primary antibodies used in this thesis (paper I and III) were the stem cell/neural progenitor markers NESTIN, VIMENTIN and SOX2 (sex determining region Y)-box 2); differentiation markers GFAP (glial fibrillary acidic protein), MAP2 (micro-tubule associated protein 2) and OLIG2 (oligodendrocyte transcription factor 2). The cells were thereafter incubated with a secondary antibody, targeted against the species of the primary antibody thus binding to it. The secondary antibody was also conjugated to an Alexa fluorophore allowing for detection of the protein targeted with the primary antibody. DAPI (4',6-diamidino-2-phenylindole) was used as a nuclear counterstain for all cells. The Operetta (PerkinElmer, Waltham, USA) was used for image capture, image analysis and automated cell quantification.

DNA methylation analysis (paper I, II, IV, V)

DNA was extracted from tumour tissue, cell pellets or FFPE tissue and thereafter bisulfite modified (Zymo, Orange, USA). Bisulfite modification converts unmethylated cytocines into uracil, whereas methylated cytosines are unaltered, thus allowing for subsequent distinction of methylated vs unmethylated cytosines. DNA extracted from FFPE samples is frequently degraded and unsuitable for array-based processes[228-230]. The bisulfite-modified DNA from FFPE samples was therefore restored/repaired using the Infinium FFPE restore kit (Illumina, San Diego, USA) prior to array processing.

DNA methylation was measured for all samples with Infinium BeadChip arrays (Illumina) evaluating >450 000 (450K array) or 850 000 CpG sites (EPIC array) respectively. The human genome contains in total almost 30 million CpG sites[130], and the CpG sites selected on the arrays include CpG islands, open sea, gene promoter, intergenic regions, enhancers etcetera[231]. For each CpG site on the methylation array, there are beads, which emit different colours (red and green) depending on the methylation state of the loci. We chose methylation arrays since they are the most common in the field, there exists many publically available datasets and packages, and most important, the clinically used tumour classifier is based on this array[155].

The raw DNA methylation data from the arrays was processed using the statistical software R (https://r-project.org) with the R package ChAMP[232]. The methylation levels were calculated as β-values:

$$\beta = \frac{\max(y_{meth})}{max(y_{meth}) + max(y_{unmeth}) + \alpha}$$

where α is a constant to avoid a small denominator. β ranges between 0, completely unmethylated, and 1, completely methylated.

The methylation data was thereafter normalised using the minfi[233] and ChAMP[232] packages. CpG sites located on sex chromosomes, single nucleotide polymorphism sites, aligning to multiple sites or probes with missing values were filtered away. Differentially methylated positions (DMP) were identified as sites with a $\Delta\beta \geq 0.3$ (paper IV and V) between two samples. In paper I and II, we also looked at major methylation changes, i.e. a switch from methylated to

unmethylated or vice versa, and for that purpose used a threshold of $\Delta\beta \geq 0.51$, as defined previously[234,235].

CNAs were inferred from the methylation array data using the conumee package[236]. Methylation-based classification and subclassification of the samples were done on the array data using the MethPed classifier[167] (paper I and II) and the MNP classifier[155] (paper IV and V). MNP classifier version 11.b4 was used in paper IV and the new 12.5 version in paper V. Version 11.b4 classifies the samples first into a methylation class and then into a subclass. Version 12.5 classifies the samples first into a methylation superfamily, then a family, followed by the class and subclass. The classifications are given a calibrated score (ranging between 0 and 1) reflecting the confidence in the classification[155]. Version 11.b4 had a recommended threshold of calibrated score ≥0.9 for the methylation class, and subclass calibrated score ≥0.5, whereas 12.5 employs ≥0.9 at all levels. Scores below the threshold can still be useful, but require an integrated assessment[168].

The Horvath methylation age estimator[174,175,177] was used in paper IV and it is based on the methylation values of 353 CpG sites. The Horvath methylation clock was trained on cancer and normal tissue from children and adults and modelled to reflect the chronological age.

Statistical methods (paper I-V)

Pearson correlation was used to determine the linear association of two variables, ranging from -1 to 1. Student's t-test was used to determine if the means of two variables differed significantly. If nothing else is stated, a p-value of <0.05 was considered significant. Bonferroni or Benjamini-Hochberg correction was applied for multiple testing. Kaplan-Meier analysis was used in paper II to visualize the proportion of injected animals alive and the time-points for their deaths/euthanisation. Hierarchical clustering was used on methylation data as it groups the most similar samples into a cluster based on a distance metric of their methylation values. The hierarchical algorithm continuously merges the two closest clusters and visualises the results in a dendrogram.

Results and discussion

Paper I: Stem cell cultures derived from paediatric brain tumours accurately model the originating tumours

The prognosis for children with GBM is devastating[25] and functional studies are desperately needed to improve the understanding of the cellular and molecular pathogenesis, and to develop new therapies. Unfortunately, well-characterised model systems for paediatric brain tumours are scarce, and utilising model systems for adult brain tumours is unsuitable given the molecular differences (as discussed above) between adult and paediatric brain tumours[11,14,16,237]. The aim of paper I was therefore to establish a stable *in vitro* cell culture system that closely resembles the originating paediatric GBM tumours.

We successfully established cell lines from six paediatric GBM tumours and showed that the cells were proliferative for at least 30 passages, expressed NSC markers (**Fig. 7A**), could grow as neurospheres, responded to differentiation cues, and initiated tumours upon injection into mice (**Fig. 7B**). This suggests that the cells are in fact CSC according to the criteria set forth by the American Association of Cancer Research[80]. The cells largely retained the CNAs and point mutations of the tumour and displayed a high similarity in DNA methylation pattern to its originating tumour (**Fig. 7C**). In fact, less than 3% of the investigated CpG sites changed methylation state (unmethylated to methylated or vice versa) in the cells compared to the originating tumour.

That the cells are representative of the originating tumour is key for all functional studies, and one of the major advantages of patient-derived cells over traditional cell lines. Traditional cell lines have often been cultured for decades in serum and may no longer represent the tumour they were derived from[82,92,96]. In a worst-case scenario, they may not actually be the tumour type of interest due to a mix-up, as was the case with the adult GBM cell line U87[238]. Our culture system is serum-free and the addition of serum induced differentiation. It should be mentioned that, similar to the adult setting, cell lines cannot be established from all GBM[102,239]. The reason why is not completely understood, but it has been shown that adult

GBM patients from which a cell line could be established, had shorter survival than those where a cell line could not be established[102], suggesting that the culture conditions are more suitable for aggressive tumours. Lower-grade tumours cannot be established either under the conditions we used, possibly because of a lack of a CSC population or due to their less aggressive nature.

To summarize, the established cell lines fulfilled the criteria of CSC and were similar to the originating tumours in terms of methylation pattern and genomic aberrations. We thus concluded that we have a robust model system of CSC where the cells accurately represent the tumours they were derived from.

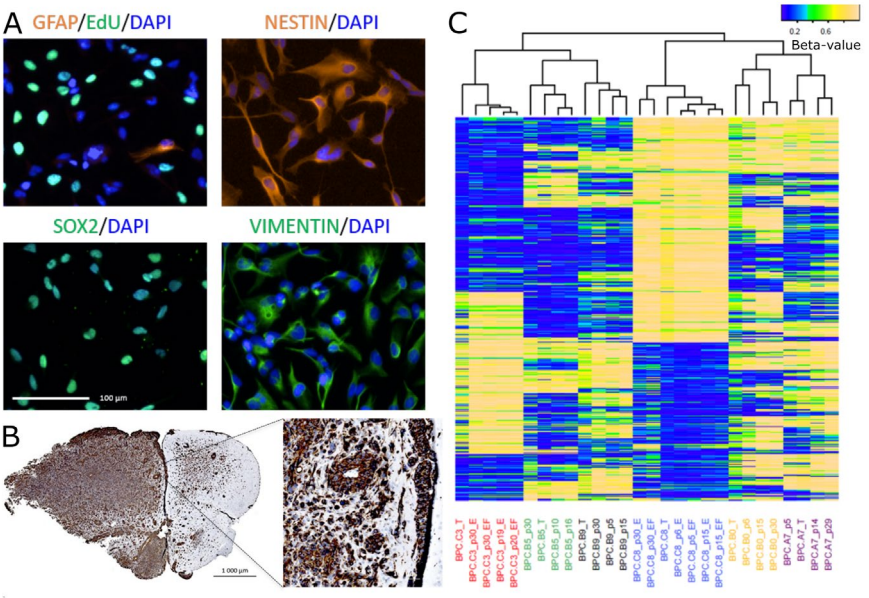

Figure 7. Characteristics of established cell lines*. A) The cells are proliferative (visualised with EdU incorporation), express stem cell and neural stem cell markers (NESTIN, SOX2 and VIMENTIN), and B) initiate brain tumours upon orthotopical transplantation into mice. Brown colour is staining by a human-specific antibody for NESTIN. C) Unsupervised clustering of the β-values of the top 5% variable methylation sites clusters all cell lines together with their originating tumour, thus demonstrating their similarity. The colour of the samples denote their patient identity. The figures are from Paper I.*

Paper II: Cell line-based mouse model mirrors the clinical course of the patient

In paper II we wanted to further characterise the tumours that formed in mice after injection by the three CSC lines described in paper I. We injected three additional CSC lines and all six were evaluated for tumour formation in mice and compared to the originating patient tumour and the *in vitro* cell line. At the time, this was the first cell line-based xenograft mouse model of paediatric GBM. Other described models injected tumour tissue or freshly dissociated cells rather than cultured adherent cells. By injecting tumour tissue there is a variability between mice in the same group and reported engraftment rates were 31% (8 of 26)[104], 67% (2 of 3)[240] or even complete failure (0%)[241]. We therefore transplanted cultured CSC in relatively low passages (~passage 10), and all six CSC lines formed tumours in mice with an engraftment rate of 97% (29/30 mice), which far supersedes the models based on injection of tumour tissue or dissociated tumour cells.

The survival time of the injected mice varied between the cell lines, but importantly, was similar for replicate mice injected with the same cell line (**Fig. 8A**), demonstrating a repeatability and homogeneous growth. All tumours in the mice were classified as GBM by the methylation-based classifier MethPed[167]. The methylation pattern in the xenografts was very similar to both the injected CSC and the originating patient tumours (**Fig. 8B**) as very few DMPs were observed. The CSC thus retain their methylation profile *in vivo*. Similarly, few de novo CNAs in the xenograft tumours compared to the injected CSC were identified, suggesting that the genome is mostly stable.

The formed tumours were evaluated by histology and shown to be very infiltrative, and presented similarities to the original patient tumours. Interestingly, the survival time of the mice injected with CSC correlated significantly to the survival time of the patients from whom the CSC were derived (**Fig. 8C**). This shows that the mouse model mirrors the clinical course of the patient and is therefore a representative model for evaluating new treatments. It is a major advantage compared to previously published models and by injecting cells rather than tumour tissue, we obtained a high engraftment-rate and homogeneous replicates without needing to passage mice to maintain the model. This is in line with the ethical animal 3R guidelines of reducing, refining and replacing the use of animals.

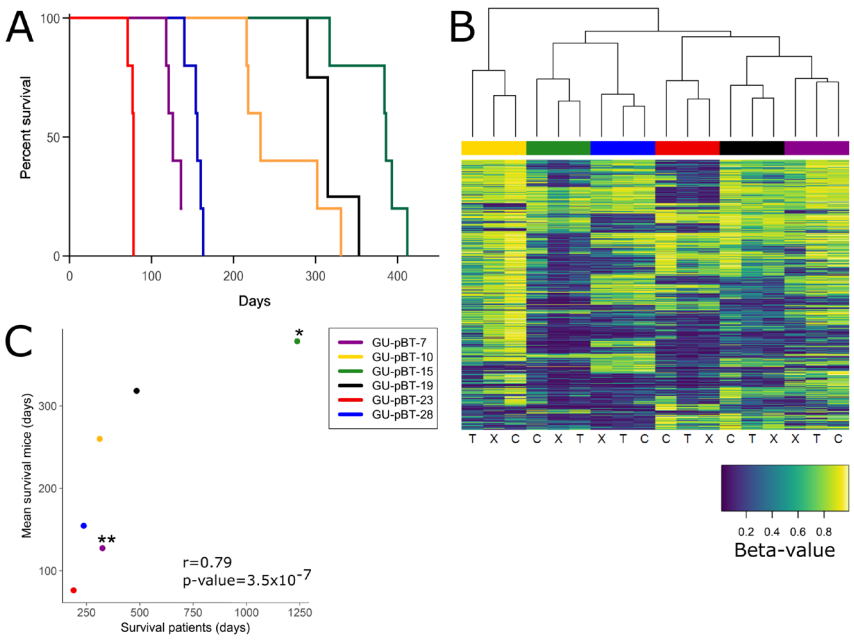

Figure 8. Mouse model mirrors the clinical course of patients*. A) Kaplan-Meier survival curve of the injected mice (5 per cell line). B) The xenograft tumour (X) retained the methylation pattern of the injected cell line (denoted C) and the originating patient tumour (T). C) The mean survival of the mice correlated significantly with the survival of the patients from whom the cells were derived. The figures are from Paper II.*

No model is perfect and disadvantages of this model include that it is based on injecting *in vitro* cultured cells, which, as described above, is also a great advantage. However, it limits the model to tumours from which cell lines can be derived and that is not the case for all, particularly not lower-grade. Further, immunodeficient mice are used to allow for the injection of human material, thus precluding analyses of the immune system. Another drawback is the difficulty in following tumour growth in real time and MRI was not successful in detecting the tumour formation, likely due to the infiltrative nature of GBM. This obstacle could be overcome with e.g. luciferase labelling of the cells prior to injection, enabling *in vivo* imaging during the experiment, likely allowing for shorter experiments.

Despite the limitations, which apply to most paediatric GBM mouse models, we have created an *in vivo* model where established cell lines are injected orthotopically with a very high engraftment rate (97%). The xenograft tumour is representative of the patient tumour and mirrors the clinical course of the patient.

Paper III: CRISPR knockout screen identifies essential genes in paediatric cancer stem cells

The prognosis for high-grade tumours such as GBM is poor and there is no consensus on treatment for these patients[7,25]. New treatments are thus needed to improve survival and reduce the severe side effects to the still developing brain that the current treatments (surgery, radiation and chemotherapy) inflict[27,242,243]. The aim of this project was therefore to identify new treatment targets in paediatric GBM by performing a CRISPR-Cas9 knockout screen on our patient-derived CSC with an epigenetic/chromatin modifier library. A genome-wide screen was, at the time, considered to require too many cells to be feasible for our patient-derived system. We therefore selected a focused epigenetic/chromatin modifier library given the importance of epigenetic deregulation in childhood cancer[10,14,22,237].

The results from the knockout screen showed that the screen robustly identified the positive controls and previously known essential genes. Comparisons to previous data sets with knockout screens[213] were then made to identify novel genes in GBM that are potentially sparing for normal cells. We thus excluded some of the top hits as they were well-known and predicted to be essential in normal cells as well. The ideal target for therapy would be a gene (protein) essential only for the cancer cells (including the CSC), but not for healthy normal cells, which would then be spared by the treatment.

After exclusion, we selected a handful of hits for individual validation in the CSC through nucleofection of RNPs. Knockout through this procedure yielded high cleavage efficiencies similar to previously reported[244,245]. The individual knockout of the genes significantly decreased the cell growth (proliferation and viability) of the CSC, in concordance with the screen results. Importantly, we also verified that there were no off-target effects. The choice of RNPs[202,204,205], and gRNA designed to minimize off-target effects[203] likely contributed.

The genes were also knocked out in NSC with less effect, indicating that the genes are more important for the growth of CSC. This demonstrated that the genes are essential for CSC growth and potential treatment targets. One of the genes had a commercially available inhibitor, which we employed on the CSC and it killed virtually all cells after only a few days. We therefore suggest that the validated genes should be further evaluated as treatment targets in paediatric GBM.

Paper IV: Intratumour methylation heterogeneity is a feature of adult glioblastoma

Intratumour heterogeneity in GBM tumours on a transcriptomic and genomic level is well-documented[71-73]. We therefore aimed to determine whether it also exists on the epigenetic level, and if this affects DNA methylation-based classification and biomarkers. We therefore sampled spatially separated biopsies from 12 adult GBM patients, processed the samples on DNA methylation arrays and used the MNP DNA methylation classifier v11b4[155] to obtain diagnoses and subclasses, as well as analysed the number of DMPs within tumours. Inter- and intra-observer bias regarding classification based on histology is an issue[163], whereas an unbiased diagnosis can be obtained from the methylation-based classifier. Methylation classification is already now included in the current CNS WHO 2021 classification[29] for certain tumours (e.g. subgrouping of medulloblastoma), and used clinically for paediatric CNS patients in several countries[168-170]. Its diagnostic value in adult CNS tumours, particularly for tumours with unusual histology or inconclusive molecular profile, has also been demonstrated[246].

All samples in this study were classified as GBM by the methylation classifier, but the GBM subclass differed within five out of 12 tumours (**Fig. 9A**). A subsequent study later reported four of 27 adult GBM with subclass heterogeneity[247]. The results from the MNP classifier suggest that biopsies from different GBM tumours can be more similar to each other (based on the CpG sites defining the subclasses), as they have the same subclass, compared to biopsies from the same tumour (that have another subclass). It is possible that the observed intratumour subclass switch is a sign that further refinement of the subclasses is needed. We recently analysed another cohort of GBM samples with the newest MNP classifier version (v12.5) with the same thresholds as above and detected three out of 10 GBM tumours with intratumour subclass heterogeneity, indicating that heterogeneity is the cause of the subclass switch (or that additional refinements are needed). It should also be noted that the GBM subclasses are not clinically used today and have no impact on treatment decision, but it is plausible that they will be in the future, in which case the subclass heterogeneity should be addressed.

We also noted a high number of CpG sites that differed in methylation values ($\Delta\beta>0.3$) within the GBM tumours; on average 17 000 sites (range: 500-51 000; **Fig. 9B**). This can be compared to meningioma CNS grade I tumours, which only

had on average 110 sites (range: 90-130) that differed intratumourally. This further shows the intratumour heterogeneity in GBM regarding the methylation pattern. One aspect to take into account when comparing different regions of a tumour is that the tumour cell content can vary and as a result affect the methylation pattern. We therefore had all samples evaluated for histology by a specialist in neuropathology and excluded samples with <70% tumour cell content. Also, we estimated the tumour cell content based on the methylation values and used it to deconvolute the methylation data accounting for tumour purity. The obtained results were similar as described above, suggesting that differences in tumour purity was not driving the observed heterogeneity.

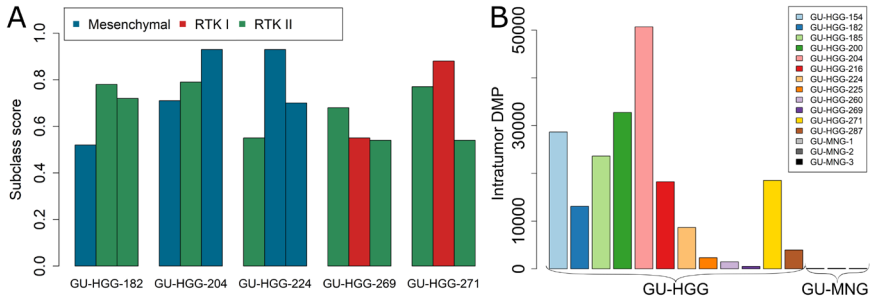

Figure 9. Intratumour methylation heterogeneity in adult glioblastoma*. A) Multiple methylation subclasses exist within adult glioblastoma (GBM) according to the MNP methylation-based classifier. B). Many CpG sites are differentially methylated (DMP) within the GBM tumours (average 17 000) whereas almost no DMP exist within the low-grade meningioma (MNG; average 110). The figures are from Paper IV.*

The alterations within GBM tumours were significantly enriched in open sea regions (CpG sites located >4kb from a CpG island) and reduced mainly in CpG islands, but the alterations did not seem to occur in specific sites in these regions. One of the genes that were affected in GBM was *MGMT* (1 of 12 patients), which is used clinically for treatment allocation in elderly GBM patients. This means that patients could receive a different treatment depending on the region of the tumour that was sampled, and thus warrants further studies. We conclude that intratumour DNA methylation heterogeneity is a feature of adult GBM, and the observed heterogeneity needs to be considered for future clinical use of methylation-based biomarkers and subclasses.

Paper V: Methylation alterations in paediatric brain tumours do not affect methylation-based classification

Given our findings of intratumour heterogeneity and multiple subclasses in adult GBM in paper IV, we asked whether the same was true in the paediatric setting. We included patients operated for a CNS tumour and sampled tumour tissue from multiple spatially separated regions. We also included paired primary and relapse samples to assess temporal heterogeneity. All samples were classified with the MNP methylation-based classifier v12.5[155]. Unlike the results in paper IV, the methylation subclass was homogeneous in our cohort of paediatric brain tumours, both in the spatial and temporal setting. Thus, we found no risk for sampling bias, but further studies with larger cohorts and additional diagnoses are required. However, the results are reassuring since methylation profiling is used clinically for paediatric brain tumour in several countries[168-170].

Even though the subclassification was homogeneous, some methylation alterations (DMPs) occurred both spatially and temporally in the tumours. The alterations were more frequent in higher-grade tumours, and increased with longer times between the primary and relapse tumour. This could be because the tumour had longer time to gain alterations. High-grade tumours generally acquire more CNAs and it is therefore unsurprising that the same is true for methylation alterations. The alterations were not shared between the patients, and mainly located in OpenSea regions (as seen in paper IV also), suggesting that the methylation changes occur at random CpG sites. The methylation alterations could potentially be viewed as "passenger" alterations, which more easily occur in less conserved regions (OpenSea) compared to CpG islands that are more conserved. These alterations are nonetheless of interest to characterise as they could alter a methylation biomarker, e.g. *MGMT* as seen in the adult setting (paper IV), or the methylation subclass if enough of the defining CpG sites are altered and with large enough changes in methylation value. This was, however, not the case in the paediatric CNS tumours in our study, suggesting a robust classifier, and it is likely assisted by the high threshold required for a successful classification by this version. Samples not reaching the threshold may still be valid, but an integrated assessment of the methylation classification, histology, CNAs and the pathological anatomical diagnosis is necessary to establish if the classification is plausible[29,168,246]. In conclusion, methylation classification and subclassification are homogeneous in paediatric brain tumours spatially and temporally.

Concluding remarks and future perspective

Pre-clinical model systems for childhood cancer

Over the last decade, through various molecular analyses, it has become apparent that paediatric tumours differ substantially from their adult counterparts[10,11,16], and that dedicated research on this patient group is required. As a result, childhood cancer research has increased, e.g. through The Innovative Therapies for Children with Cancer (ITCC) consortium[248-250] aiming to develop novel therapies, precision medicine through the INFORM (INdividualised therapy FOr Relapsed Malignancies)[251,252] and paediatric MATCH (Molecular Analysis for Therapeutic CHoice)[253] studies, and identification of genetic dependencies in childhood cancers (paediatric DEPMAP)[216].

Very few inhibitors make it all the way through clinical trials into clinical use and benefit of childhood cancer patients. One of the main reasons is the lack of representative model systems, leading to pre-clinical research in traditional serum-cultured cell lines that do not accurately model the disease. Further, many paediatric tumours (e.g. GBM) are rare and enrolment into clinical trials is therefore a challenge. Both of these issues highlight the importance of international collaboration/clinical trials and the pressing need for model systems representative of the paediatric cancer biology. In paper I and II, we therefore established a patient-derived *in vitro* and *in vivo* model system of paediatric GBM. Importantly, the cells accurately represented the patient tumour they were derived from. Injection of the cultured cells orthotopically into mice yielded a high engraftment-rate (97%), and the xenograft tumours were similar to the patient tumours in terms of histology, methylation pattern and survival.

Model systems used in future research will likely move away from the traditional cell lines in benefit of primary patient-derived models. Further, cell models cultured on 3D scaffolds, as spheroids or organoids[254-256] will likely increase in popularity. They provide a more accurate model of the complexity of an organ and cell-cell interaction compared to the artificial two-dimensional environment of adherent cell culture. For brain tumours in particular, there is a great need of

modelling the blood-brain barrier as it limits the delivery of therapeutic substances to the brain. *In vitro* models based on biomaterials and 3D printing has been suggested as well as mouse models[257], where so called "humanized mouse models" are gaining traction. This model is based on engraftment of human tumour cells and immune cells in an immunodeficient animal, which gains a subset of the human immune system[258]. This model is not without its challenges[259], but holds great potential for future *in vivo* cancer models.

CRISPR screens to identify essential genes in cancers

The CRISPR community has grown tremendously since its discovery in 2012[180] and now, it has won the Nobel Prize (2020) and grown into a well-known and crucial method in life science[184,187]. There is an ever-growing number of new CRISPR-based methods, for instance base editing[260,261], removing the need for a double-stranded cut in the DNA, and modulation of epigenetic features (methylation, demethylation etcetera) through dCas9[200,201]. There are also several ongoing clinical trials in human cancers in e.g. lymphoma and leukaemia with transfusion of *ex vivo* CRISPR-edited T cells (ClinicalTrials.gov identifier: NCT05066165 and NCT04035434). Clinical trials have also been performed where the CRISPR edit occurs in the human body[185]. Even though the initial reports of massive off-target effects at random locations back in 2018 were false and later retracted[262-267], questions and ethical concerns still remain regarding off-target effects in humans[186] and need to be further investigated.

The large-scale knockout screens performed in cancer cells is an excellent example of the utility of CRISPR in basic science to identify dependency genes in various cancers[213,215,216]. Unfortunately, these screens have mainly used traditional cell lines cultured in serum. Our knockout screen in paper III, however, used our cell cultures (established in paper I) representative of the patient tumour. We identified many novel genes essential for the growth of CSC in paediatric GBM, and validated several by individual knockout. We conclude that these genes are novel treatment targets in childhood GBM and should be further investigated for clinical use.

Methylation profiling for improved diagnostics

Deregulated epigenetics was included as an emerging hallmark of cancer in the 2022 revision[3], highlighting its involvement in cancer. This thesis has incorporated deregulated epigenetics in all five papers. Paper I-II used it to verify the similarities of the established cell lines/mouse models to the patient tumour. Paper III employed an epigenetic/chromatin modifier library for the knockout screen. Finally, paper IV-V, focused on the diagnostic properties of methylation-based classification.

Methylation-based classification has proven more accurate than histopathological analysis, allowing for further stratification into subclasses (e.g. within medulloblastoma and ependymoma), and discovering novel tumour entities[42,155,165]. It has subsequently been integrated in diagnostics for all paediatric brain tumours, and for challenging cases of adult brain tumours in several countries[29,168,170,246]. In paper IV, we concluded that there is a high intratumour methylation heterogeneity in adult GBM with multiple methylation subclasses residing within the same tumour and differing *MGMT* status. While these subclasses, as of now, are not involved in treatment decisions, the *MGMT* methylation status is and needs to be considered. The strict cut-off at 9% methylation for unmethylated vs methylated *MGMT* has been questioned[268,269], and a large meta-analysis provided no strong evidence regarding the optimal level[270]. Further, it is plausible to imagine a future where GBM subclasses (in their current form or remade/refined) are introduced clinically, in which case the subclass heterogeneity needs to be addressed.

Paper V showed that, unlike the adult GBM in paper IV, paediatric brain tumours had homogeneous methylation-based subclassification, both spatially, within the same tumour, and temporally (primary vs relapse tumour). However, methylation alterations did occur and they increased over time and in more aggressive tumours. The alterations were enriched in regions not affiliated with genes and far from CpG islands, suggesting that they occur in less conserved regions. We concluded that these alterations did not affect the methylation-based classification in paediatric brain tumours. This is essential since methylation profiling is already used clinically for diagnosis in multiple countries, including Sweden, and more will follow. The clinical use will likely expand to routinely include adult brain tumours as well. Subsequent updates of the classifier will probably contain novel tumour

entities and subclasses, and even expansion to other tumour types (e.g. a sarcoma classifier exists already[271]).

Over the last few decades, the severity of the treatment-induced side effects on paediatric brain tumour survivors have become apparent and increased the focus on quality of life after treatment, not survival at any cost. The identification of molecular methylation-based subgroups within e.g. medulloblastoma and ependymoma, as described above, provides the opportunity of tailoring the treatment based on the prognosis. Subgroups with poor prognosis can be given aggressive treatment whereas subgroups with good prognosis can receive less aggressive treatment and reduce the side effects.

Another advancement in the field would be a classifier using cell-free DNA (cfDNA) from liquid biopsies (e.g. serum, cerebrospinal fluid, urine). cfDNA is extracellular DNA released from cells (tumour and normal cells) into body fluids, where cfDNA has been shown to reflect the tumour genome it was shed from[272-275]. A "liquid biopsy" classifier would thus allow for non-invasive classification of tumours, whereas today's classifiers require a tissue sample (obtained through tumour resection or biopsy). Hence, cfDNA could be used for diagnostic purposes also for inoperable patients, and to follow the disease progression. There has been development towards such classifiers that were reported to successfully distinguish gliomas from other tumour types[276-278] and determine the medulloblastoma subgroup[279], based on the cfDNA methylation profile. These are promising first steps and will be interesting to follow over the next few years.

Overall, this thesis provides a small piece to the puzzle of the workings of deregulated epigenetics and CSC in brain tumours, bringing us closer to the goal of improved diagnostics and cancer treatment for patients with brain tumours.

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