Talking Neurons: The key feature of chemical synapses is the presence of small organelles called synaptic vesicles within the presynaptic terminal. These spherical organelles are filled with neurotransmitters, the chemical signals secreted from the presynaptic neuron, and it is these chemical agents which are needed as messengers to make neurons communicate with each other.
General discussion and future perspectives
Medulloblastoma and glioblastoma are the most common high-grade brain tumors in children and adults respectively. In recent years our knowledge on the molecular background of these high-grade brain tumors has increased extensively. Despite the increase in this knowledge, outcome in medulloblastoma and glioblastoma remains poor and survivors suffer from severe long-term treatment associated side effects. This thesis was aimed at identifying promising treatment targets for medulloblastoma and glioblastoma, by focusing on three different processes driving high-grade brain tumors: 1) developmental pathways, 2) angiogenesis, and 3) therapy resistance. Here, results from these three projects will be discussed, together with suggestions for further research and improvements.

Trimethylation of H3K27 during cerebellar development and in medulloblastoma

Medulloblastoma is considered to encompass a collection of four clinically and molecularly distinct tumor subgroups, i.e. WNT, SHH, Group 3 and 4. Extensive studies have provided compelling evidence that medulloblastoma is caused by aberrantly dividing precursor cells present during (pre)cerebellar development. Mutations in developmental pathways, like the SHH pathway and the WNT pathway have been shown to drive medulloblastoma formation in specific subgroups. Histone modifications are increasingly being recognized as key events during cerebellum development and medulloblastoma tumorigenesis. In Chapter 2 we have investigated the trimethylation status of histone H3 lysine 27 (H3K27), as well as the expression of the H3K27 methylase EZH2 and demethylase KDM6B, during human cerebellum development and in medulloblastoma.

Recent studies show that deregulated post-translational modifications of H3K27 are common aberrations in medulloblastoma. More importantly, high H3K27 trimethylation is associated with a dismal outcome, particularly in Group 3 and 4 medulloblastoma. Because the four subgroups seem to originate from different precursor cells present during cerebellar development, we extended our study by describing the trimethylation status of H3K27 during human cerebellum development. We have provided evidence that during development of the human cerebellum H3K27 trimethylation and its regulators are expressed in a spatio-temporal manner. And in line with previous studies, an aberrant expression pattern was seen in medulloblastoma, compared to normal human cerebellum.

Thus fare, two demethylases for H3K27 trimethylation have been identified; KDM6A and KDM6B. The importance of KDM6A is highlighted by the fact that this demethylase is the most common mutated H3K27 regulator in medulloblastoma. Moreover, KDM6A mutations seem to occur exclusively in Group 4 medulloblastoma. Unfortunately, we did not include KDM6A expression in our study, since no reliable antibody for KDM6A immunohistochemistry was available. However, with the recent description of an antibody...
against KDM6A for immunohistochemistry\textsuperscript{14}, it will be important to reanalyze in our cohort of human cerebellum the expression of KDM6A.

We determined the status of H3K27 trimethylation and expression of EZH2 and KDM6B in only two primary medulloblastoma samples, for which we also had no information on the subgroups they belonged to. The four medulloblastoma subgroups seem to originate from different precursor cells and each harbor unique subgroup specific mutations and gene signatures. To correlate results from our developmental expression studies with the different medulloblastoma subgroups, we should extent the number of medulloblastoma samples investigated, including appropriate numbers of each subgroup. Dubuc \textit{et al.}, have previously investigated the H3K27 trimethylation mark in a large cohort of medulloblastoma samples\textsuperscript{11}. However, they did not focus on the protein expression of the regulators of this histone mark. In ongoing studies we have extended our expression studies by including medulloblastoma samples from different subgroups and by addressing the expression of KDM6A in medulloblastoma and during human cerebellum development.

The main limitation of our developmental study has been the descriptive nature of our analyses. We have only determined the expression patterns of H3K27 trimethylation, EZH2 and KDM6B during development. However, by showing for the first time that H3K27 regulators are expressed in a spatio-temporal manner during human cerebellar development, we have paved the way for future research in which more in depth studies can be carried out looking at H3K27 trimethylation during different stages of cerebellar development and linking them to medulloblastoma tumorigenesis.

\textbf{EZH2 and microRNA-101 signaling in glioblastoma angiogenesis}

Glioblastoma typically invades the brain parenchyma and is notorious for its capacity to induce angiogenesis, the process of sprouting new blood vessels from pre-existent vessels\textsuperscript{15}. Glioblastoma cells produce large amounts of the pro-angiogenic vascular endothelial growth factor (VEGF) under hypoxic conditions. The secreted VEGF interacts with endothelial cells comprising the glioblastoma vasculature, causing endothelial cell survival, proliferation, and sprouting\textsuperscript{16}. Epigenetic and post-transcriptional modification, through histone modification and microRNAs (miRs), have emerged as potential regulators of angiogenesis\textsuperscript{17,18}. In chapters 3 and 4 we have investigated the role of the histone methyltransferase EZH2 and miR-101 signaling in the regulation of gene expression in endothelial cells and glioblastoma cells during angiogenesis.

First, we have demonstrated that miR-101 is down-regulated in glioblastoma, resulting in impaired translational repression of EZH2. Overexpression of EZH2 induces tumor progression through proliferation, migration/invasion, and angiogenesis. To further investigate the role of EZH2 and miR-101 in glioblastoma angiogenesis, we then focused on the interaction between glioblastoma cells and tumor-associated endothelial cells. We provide evidence
that glioblastoma cells produce VEGF, which down regulates miR-101 in tumor-associated endothelial cells. The reduced miR-101 expression results in increased expression of EZH2 in endothelial cells, which partly causes a pro-angiogenic effect.

Overexpression of EZH2 has been reported in several types of cancers, and is regulated at transcriptional, post-transcriptional and post-translational levels\(^{19-22}\). In our studies we focused on the post-transcriptional effect of miRNAs on EZH2 expression. From the four miRNAs we identified to potentially target EZH2 expression and which were down regulated in glioblastoma as compared to normal brain, we focused on miR-101. Since miR-101 was found to target EZH2 in other tumors\(^{23,24}\) and was confirmed to bind the EZH2 3′-UTR at two sites, it seemed to be the most promising target. Moreover, down regulation of miR-101 in glioblastoma seems to be caused partly by genomic loss of its loci in approximately 20% of glioblastoma cases\(^{23,25}\). Recently, miR-138 and miR-708 were also found to regulate EZH2 expression in glioblastoma\(^{26,27}\). In other tumors miR-25, miR-26, miR-30d, miR-98, miR-137 and miR-214 have shown to regulate EZH2\(^{28-33}\). Whether these miRNAs regulate EZH2 in glioblastoma needs to be established.

To mimic the behavior and signaling in tumor-associated endothelial cells we have used primary microvascular endothelial cells isolated from human brain (HBMVECs) in co-culture with the glioblastoma cell line U87. Whether this model genuinely represents tumor-associated endothelial cells in glioblastoma is a point of concern. First, the use of a single tumor cell line to recapitulate the heterogeneous character of glioblastoma seems unlikely. In vitro culturing of tumor cells causes significant genetic changes when compared to their primary samples\(^{34,35}\). Similarly, HBMVECs might not fully recapitulate the behavior of tumor-associated endothelial cells\(^{36}\). Recently, a subpopulation of glioblastoma cells, the so-called GBM stem-like cells, have shown to be able to differentiate into endothelium cells\(^{37,38}\). Second, the role of vascular pericytes has not been addressed in this thesis. Vascular pericytes play essential roles to maintain functional vessels to support tumor growth\(^{39}\). The recent discovery that the majority of vascular pericytes in glioblastoma are derived from GBM stem-like cells\(^{40}\) adds to the complex interplay between tumors cells, tumor-associated endothelium cells and vascular pericytes. Until recently, alternatives to this reproducible co-culture system were not available. However, with the development of stem cell-derived three-dimensional cultures, so-called organoids, we can more closely mimic the complex niche in which glioblastoma resides\(^{41-43}\).

In our experiments we have used the EZH2 inhibitor 3-deazaneplanocin A (DZNep), a S-adenosylhomocysteine hydrolase inhibitor, that inhibits methyltransferases and induces degradation of EZH2\(^{44}\). However, clinical implementation of DZNep seems limited. DZNep is a non-selective EZH2 inhibitor which has been found to inhibit the methylation of other histone lysines and arginines\(^{45}\). Moreover, DZNep does not cross the blood brain barrier, limiting its therapeutic potential in patients with glioblastoma\(^{46}\). Fortunately, novel and more
selective EZH2 inhibitors have recently been developed\textsuperscript{47–50}. These inhibitors however, still need to prove their potential in preclinical and future clinical studies. For our in vivo experiments we have used a subcutaneous mouse model due to the inability of DZNep to cross the blood brain barrier. Unlike glioblastoma, subcutaneous tumors are characterized by a well-demarcated non-infiltrative mass, and are perfused by leaky vessels making them much more accessible to systemically administrated drugs\textsuperscript{51}. For a more reliable clinical translation of our results, the use of an orthotopic glioblastoma model would have been more informative, as these models have been shown to more closely mimic the infiltrative and angiogenic behavior of human glioblastoma\textsuperscript{52}.

**WEE1 protects glioblastoma against mitotic catastrophe**

Glioblastoma is one of the most aggressive tumors due to its inherent resistance to both chemo- and radiotherapy\textsuperscript{15}. Pathways causing resistance to conventional therapy were focus of chapters 5 and 6. Kinases have emerged as important targets in cancer as they have been found to regulate crucial cellular functions, including those responsible for therapy resistance\textsuperscript{53}. By determining kinase expression in publically available gene expression data sets we identified a glioblastoma-specific kinase expression profile. Nine kinases were found to be substantially and frequently overexpressed in glioblastoma, with the WEE1 kinase spearheading this list. Moreover, WEE1 overexpression was correlated with poor outcome in glioblastoma. After DNA damage induction, glioblastoma cells mainly arrest in the G\textsubscript{2} phase, due to an impaired G1 checkpoint. The resistance of glioblastoma cells to chemo- and radiotherapy is partly due to their proficient ability to repair treatment-induced DNA damage during this G\textsubscript{2} cell cycle arrest. We demonstrated that WEE1 is the major regulator of the G\textsubscript{2} checkpoint in glioblastoma. Inhibition of WEE1 by siRNA or a small molecular compound in tumor cells exposed to DNA damaging agents resulted in abrogation of the G\textsubscript{2} arrest, which caused premature termination of DNA repair, and eventually leading to mitotic catastrophe and cell death. Here, we have provided evidence that ‘pushing’ tumor cells into cell division seems to be a valid approach to overcome therapy resistance in glioblastoma. The majority of kinase inhibitors developed thus far are aimed at halting tumor growth\textsuperscript{54}. However, results from our study stresses the importance of proper timing of treatment when combining kinase inhibitors with DNA damage-induction.

When translating research results from bench to bedside, it is critical that the used model systems recapitulate the clinical setting as much as possible. Therefore, we have used two different orthotopic glioblastoma models for our in vivo experiments, including the highly infiltrative E98 model. These models more closely resemble the infiltrative and angiogenic character of human glioblastoma\textsuperscript{52}. Limitations are however also present. In our in vivo experiments the mice were treated with only a single dose of irradiation, whereas patients are treated with fractionated doses of irradiation. Therefore, it would be interesting to
repeat our in vivo experiments using a fractionated irradiation regimen in combination with a WEE1 inhibitor. A number of small molecule compounds can inhibit WEE1\textsuperscript{55–59}. For our studies we have used the WEE1 inhibitor PD166285. This WEE1 inhibitor however, has shown to be a potent but non-selective inhibitor of WEE1. Other kinase targets of PD166285 are c-Src, MYT1, EGFR, FGFR1, CHK1, and PDGFR\textbeta\textsuperscript{59}. Whether the preclinical response we showed in our studies might be due to off-target effects of PD166285, seems unlikely. All experiments, including in vivo experiments, were carried out using siRNA against WEE1 in parallel experiments, rendering comparable results. Recently, the more selective WEE1 inhibitor, MK-1775, has entered clinical testing in multiple phase I and II trials, including trials in patients with a glioblastoma. We could not include this drug in our studies due to unavailability. However, in more recent studies we have used MK-1775, and showed that it enhances radiation sensitivity in diffuse intrinsic pontine glioma and in the highly infiltrative orthotopic glioblastoma mouse model, E98\textsuperscript{60}.

The role WEE1 seems to play in cancer varies between tumors. In line with our results, WEE1 is overexpressed in various tumors, including, breast cancer, hepatocellular carcinoma, mantle cell lymphoma, ovarian carcinoma, diffuse intrinsic pontine glioma, medulloblastoma, and mesothelioma\textsuperscript{60–65}. This overexpression results in resistance to DNA damaging agents, which can be overcome by targeting WEE1 in these tumors\textsuperscript{58,60,65,66}. In contrast, WEE1 underexpression has been described in colon cancer and non-small cell lung cancer\textsuperscript{67,68}. Patients with WEE1 negative non-small cell lung cancer had a higher recurrence rate and poorer survival\textsuperscript{68}. Moreover, normal human primary prostate epithelial cells and prostate epithelium have low expression of WEE1, with increased susceptibility to unrepaired DNA damage in mitosis\textsuperscript{69}. Whether this predisposition to DNA damage and increased genomic instability in prostate epithelium leads to transformation needs to be investigated further. Taken together, cancers with increased genomic instability, typically those with deficient p53 signaling, depend on WEE1 as their main cell cycle checkpoint in response to DNA damage to survive\textsuperscript{70}. In this context WEE1 can be considered as an oncogene. However, WEE1 can also be considered to be a tumor suppressor, the loss of which drives normal prostate epithelium and, possibly, colon and lung epithelial cells susceptible to genetic aberrations and transformation.

The possible tumor suppressive properties of WEE1 underscore the importance of patient safety when introducing WEE1 inhibitors into clinical practice. Based on current knowledge it seems reasonable to conclude that inhibition of WEE1 can be a safe therapeutic strategy. We clearly showed that only the glioblastoma cells were radiosensitive to WEE inhibition, while normal human fibroblasts and human astrocytes were both unaffected by WEE1 inhibition. This is most likely due to the fact that non-neoplastic cells which contain wild-type TP53, are equipped with a functional G\textsubscript{1} checkpoint which is independent of WEE1 regulation. Almost 90% of glioblastomas harbour TP53 mutations, which render these tumors more dependent on the G\textsubscript{2} checkpoint\textsuperscript{25}. Second, no adverse effects were detected in mice after high dose
injection of WEE1 inhibitor in our dose-effect experiment. Third, early stage clinical phase I and II trials using the WEE1 inhibitor MK-1775 are showing promising results, with limited toxicity.

**Conclusion**

High-grade brain tumors are among the most aggressive tumors in both adults and children. This thesis addresses the urgent need to identify promising treatment targets for medulloblastoma and glioblastoma. Results from this thesis suggest an important role for H3K27 trimethylation during cerebellum development, and as a potential target for future therapy in medulloblastoma. Moreover, for highly angiogenic glioblastoma targeting the EZH2 appears to be a novel approach in reducing tumor-angiogenesis. Finally, the therapy resistance in glioblastoma seems partly to be induced by WEE1 activity, and our results suggest that inhibition of WEE1 kinase holds potential as a therapeutic approach in treatment of glioblastoma.
REFERENCES


