CHAPTER XIX

SUMMARY

GLIOMA

Glioblastoma multiforme (GBM) is the most malignant variant of glioma. This tumor does not only display an extremely aggressive, invasive growth pattern, but is also very difficult to treat. With a two-year survival rate of 40% and a median survival of 12-18 months after treatment, prognosis is poor. Factors contributing to this outcome can be divided in two groups. First, current treatment options are not successful in halting tumor progression. The current standard of care includes surgical debulking of the tumor mass, followed by radiation and chemotherapy (temozolomide). However, the location of the tumor (the brain) and its invasive, root like growth pattern limit the results that can be achieved by surgery. The same limitations apply to radiotherapeutic treatment, with the nature of the surrounding tissue not allowing high enough doses of radiation to be delivered. Chemotherapeutics cannot cross the blood brain barrier efficiently and often resistance develops before the tumor is eradicated. While treatment has prolonged lifespan from 4-6 months to 12-18 months, we seem to book less progression than seen in other cancer fields.

The second complicating factor, which is closely related to minimal improvements in treatment modalities, is the highly complex and aggressive behavior of GBM tumor cells. GBM tumors are highly heterogeneous, display all kinds of anti-apoptotic escape routes, suppress the immune system, invade the surrounding parenchyma with unmatched aggressiveness and possess a whole array of tools to rearrange the extra tumoral environment to their advantage. Glioma stem cells (GSC) seem to drive this process, and, as recent research has shown, can reestablish a copy of the original tumor when transplanted into immunocompromised mice. Studies investigating the genetic profile of GBM have demonstrated further that the heterogeneity has a strong genetic component. Several genetic alterations can occur, each predisposing its bearer to a certain growth pattern and level of invasiveness and, most importantly, response to therapy. The complexity and crosstalk of intracellular pathways contribute even more to the difficulty of finding an effective treatment.

Whereas for example patients with a loss of chromosome 1p respond to a chemotherapy regimen of PVC (procarbazine, CCNU and vincristine) combined with

temozolomide, GBMs with EGFR-III amplification rarely respond to chemotherapy at all. O6- methylguanine DNA transferrase or MGMT, a DNA repair enzyme that protects cells from damage caused by ionizing radiation and alkylating agents, is currently the most powerful molecular predictor of outcome and benefit of temozolomide treatment. If MGMT methylation is present, the cells are unable to properly repair DNA damage, resulting in a relatively good response to treatment.

Based on current state of matter it can be concluded that new interventions are needed. Gene therapy was long thought to be the magical approach to cure all kind of diseases, including cancer. However, clinical trials have shown this approach has yet to overcome some major limitations in order to make a difference in the medical field. Some mayor successes have been reported, however, many attempts still fail to make a clinically relevant impact. Lack of efficient gene delivery and transfer, and the inability to achieve high enough levels of gene expression are some of the major limitations of this approach. The patient's own immune response against the allogeneic gene and its viral vector is partly responsible for this. Since inadequate expression will confound results, it is hard to judge on the efficacy of certain therapeutic gene strategies and this may partly explain the inconsistency that exists between animal studies and clinical trials.

BIOLUMINESCENCE IMAGING

In order to get a better understanding of glioma tumor biology, and to develop a more successful therapeutic approach, it is important to be able to visualize the processes taking place on cellular, molecular, and even genetic level. Bioluminescence imaging (BLI) is a technique that uses the enzymatic activity of luciferases to visualize all kind of intra- and extracellular processes. In order to emit light, a chemical conversion of the luciferase by its substrate is needed. *American Firefly luciferase* (Fluc), *Gaussia Princeps* (Gluc), *Renilla reniformis* (Rluc) and *Vargulla hilgendorfi* (Vluc) have all successfully been used as mammalian cell reporters.

BLI is extensively used in cancer. Due to the high complexity, the interactions between cells and their environment, intracellular crosstalk and the invasive nature

of GBM tumors, it is highly desirable to be able to label tumor cells and detect cancer pathways. BLI can help understanding these complex processes by visualizing “what happens” during tumorigenesis, by assessing gene activation, cell growth and behavior, tracking of cancer stem cells (CSC) and metastasis and recurrence following chemotherapy. Further, it can potentially visualize tumor cell response to certain therapeutic compounds, allowing us to see which pathways and escape mechanisms are activated and where intervention is needed. Since BLI can be used both *in vitro* and *in vivo*, it can help identifying new cancer treatments by validating experimental drugs in animal models, bridging the gap between the laboratory and the clinic.

OUTLINE

In this thesis we developed reporters, diagnostics and a 'curative strategy' for Glioma, based on the joint forces of BLI and gene therapy.
In **Chapter III** we describe the development of a new antibody based *Gaussia* luciferase blood assay. *Gaussia* luciferase differs from other luciferases in that it is secreted from the cell and can be detected in the circulation. The advantages of this characteristic are huge. Instead of having to measure BLI signal by either using a CCD camera (which is time consuming and static) or by sacrificing the animal, one can simply take an aliquot of blood and measure the Gluc signal. This allows for the *ex vivo* monitoring of *in vivo* processes in real time, which is extremely valuable when studying tumorigenesis. However, Gluc emits blue light, which is partly absorbed by pigmented molecules as hemoglobin. This limits the sensitivity of the assay, as a part of the signal is lost. To overcome this problem, we developed an alternative assay in which Gluc is captured from the blood in an antibody-mediated reaction before the signal is acquired. By elimination of the signal quenching molecules the assay showed to be over one order of magnitude more sensitive in detecting the Gluc signal. The new sensitivity standard will allow us to detect small numbers of circulating cells, early tumor metastasis and apoptosis.

In **Chapter IV** the development of a multiplex *ex vivo* blood reporter system based on the secreted alkanine phosphatase (SEAP), *Gaussia* luciferase and a secreted variant of *Vargula hilgendorfi* is described. We first characterized Vluc as a blood

reporter and multiplexed it with Gluc and SEAP to develop a triple blood reporter system to monitor three distinct biological processes. As a proof of concept, we successfully monitored the response of three different subsets of glioma cells to the chemotherapeutic agent temozolomide in the same animal. This multiplex system can be extended and applied to many different fields for simultaneous monitoring of multiple biological parameters in the same biological system.

In **Chapter V** the development of an *in vivo* triple reporter system based on *Vargula, Gaussia* and *Firefly* luciferases for sequential imaging of three different biological processes is described. We applied this system to monitor the effect of the apoptosis-inducing ligand sTRAIL (soluble Tumor necrosis factor-Related Apoptosis-Inducing Ligand) on GBM tumor cells using an adeno-associated viral AAV vector. TRAIL is only toxic to cancer cell since only these cells overexpress TRAIL death receptors. However, there also appears to be a group of tumors, including GBM, that is resistant to TRAIL-mediated apoptosis. To overcome this limitation, we identified a molecule that sensitized GBM cells for TRAIL, called lanatoside C, which is a known cardiac glycoside. Since TRAIL cannot cross the blood brain barrier, we engineered the normal brain to synthesize and secrete sTRAIL. Thereby, we created a zone of resistance against newly developed glioma, which can be treated with lanatoside C therapy. We used Vluc to monitor AAV gene delivery of sTRAIL to the healthy brain parenchyma, Gluc to monitor the binding of sTRAIL to the glioma death receptor on the tumor cells and the consequential activation of downstream events, and Fluc to measure tumor response to combined sTRAIL and lanatoside C treatment. Binding of sTRAIL on tumor cells activated downstream events leading to an initial decrease in glioma proliferation. However, this was followed by tumor re-growth through resistant cells. Co-treatment with lanatoside C sensitized the resistant subpopulation of glioma to sTRAIL-induced apoptosis as monitored by the triple reporter system. Since AAV vectors, TRAIL, and cardiac glycosides have already been used in a clinical setting, though in uncombined administration strategies, this therapeutic strategy could be easily adapted for use in humans. This work is the first demonstration of triple *in vivo* bioluminescence imaging and will have broad applicability in different fields.

In **Chapter VI** the development of better variants of *Gaussia* luciferase, that can be used in the multiplex reporter systems of **Chapter III, IV** and **V,** or by themselves, are described. Gaussia luciferase has many advantageous properties over other luciferases, including high signal intensity, favorable enzyme stability and a secretion signal, which allows for signal detection in the blood. However, current limitations of Gluc as a reported include signal quenching and absorption by pigmented molecules (as discussed in **Chapter IV**) and its flash type light emission, which results in rapid light decay and makes Gluc less suitable as cell viability marker in drug screens. When thousands of compounds need to be tested, stable light emission is required. To overcome these limitations, a library of Gluc variants was generated using directed molecular evolution and screened for relative light output, a shift in emission spectrum, and glow-type (stable) emission kinetics. Several variants with a 10-15nm shifts in emission spectrum were identified, as well as a Gluc variant yielding over 10 minutes of stable light output that can be used in high throughput applications.

Different variants of the same luciferase can be used simultaneously as long as there is enough difference in their range of light emission. In **chapter VII** we characterized a codon-optimized variant of Italian firefly luciferase (IiFluc) for mammalian gene expression and used the green and red light emission variants for *in vivo* tumor imaging. The red shifted variant showed to be a useful marker for *in vivo* tumor growth over time.

In **Chapter VIII** and **IX** a simple and sensitive assay to monitor mycoplasma contamination is described. Mycoplasma contamination in mammalian cell culture is often overlooked, yet is a serious issue that can induce a myriad of cellular changes and thereby confound results. Since glioma research heavily relies on cell culture and the effects of mycoplasma can set research projects back for years, we developed a mycoplasma detection assay based on the degradation of Gluc in the conditioned medium of contaminated cells. Whereas Gluc has a half life of > 7 days in the conditioned media of mycoplasma free cells, the half life of Gluc is tremendously decreased in the presence of mycoplasma contaminations, with the level of decline correlating to the mycoplasma infection rate. Our Gluc based mycoplasma assay proved to be more sensitive as compared to commercially available BLI assays.

In **chapter X** we took a different approach for the treatment of brain cancer: we developed a liposome as a contrast agent for MRI with a higher efficiency than the conventional liposome. Liposomes are spherical, self-closed structures formed by one or several concentric lipid bilayers with an aqueous phase inside and between the lipid bilayers. They are proven effective for the delivery of drugs and imaging agents to the tumor site. Our liposome can target cells by its biotin inclusion, it can be monitored by MRI, and the therapeutic agent can be released by ultra sound, due to its thermo-sensitivity.

In **Chapter XI** and **XII** we provided an overview of the Glioma research field, placing the work developed in this thesis in a broader setting. We reviewed current research strategies, both in experimental setting and in the clinic, discussed the translational gap that exists between those two worlds and reflected on possible future directions. Cellular therapeutic strategies (including stem cells and immuno cells) seem to provide a solution for the problems that currently halt the advances in gene therapy. Stem cells have the ability to specifically target glioma cells, both invasive and located in the tumor. Aside from the homing mechanism that selectively targets tumor cells, stem cells can effortlessly cross the BBB, are easily modified to carry therapeutic genes, have immunosuppressive properties that prevent a host immunoreaction after implantation, and seem capable of shielding therapeutics such as oncolytic viruses from the host immune response, thereby ensuring long term reservoirs of therapeutic virus at the tumor site.
Immunocells are used as vaccines to stimulate an antitumor response by the patient's own immune system. The advantage of this approach is the establishment of a sustainable tumor attack, theoretically not only eradicating the tumor, but also protecting the patient against the development of recurrences. Both stem- and immuno cell therapies are now carefully introduced in the clinic, and first results seem to indicate they are well tolerable and safe. The efficacy of these approaches is not yet optimal, but examples in other fields show that when optimization steps are taken, current limitations can be overcome. Suggestions for improvement of the field and the role for BLI in this process are discussed in **Chapter XI**, **XII** and **XIII**.