CHAPTER XIII

DISCUSSION

Part 1: Sarah Bovenberg
Part 2: Hannah Degeling

Part 1: Discussion by Sarah Bovenberg

I. CREATING THE IDEAL ANTI GLIOMA AGENT

As has been highlighted throughout this thesis, brain tumors, and GBM in particular, are very difficult types of cancer to treat. A quick search on PubMed will reveal a wide range of strategic approaches that are currently being explored, ranging from new chemotherapeutic agents and/or dose schemes, drugs, surgical techniques, antibodies, to cell- and gene therapy. Even though it is beyond the scope of this thesis to discus all of these approaches, it can be concluded that many exciting research is being done, based on ideas that theoretically should be able to destroy the tumor mass completely. However, what works on paper does not necessarily work in the lab, let alone in clinical settings. Other strategies seem to work just fine – without anyone fully understanding what causes their success. When thinking about the ideal anti-glioma agent, the following characteristics should be present:

1) The agent is highly selective to glioma cells, while causing minimal damage to the normal brain tissue
2) The agent has the ability to track down single invasive cells
3) The agent should attack the glioma cells through a range of pathways, thereby blocking tumor escape mechanisms and accounting for the heterogeneous nature of the glioma cell population
4) Its action should be continuous, until the eradication of all tumor cells is completed
5) The agent should contain a biological marker that allows for monitoring of the effect of treatment over time
6) The agent should be able to cross the blood-brain barrier
7) The agent is tailored to the individual patient

Objectively, stem cell therapy seems to hold all the right cards. However, as can be concluded from our reviews in **Chapter XI** and **XII**, we still have a long way to go before all issues are resolved. This raises the following questions:

- What is needed to improve treatment efficacy?
- What is needed to bridge the gap to the clinic?

The primary aim of this thesis was to gain better understanding of both glioma biology and anticancer mechanisms by visualizing what happens at cellular and molecular level. Imaging tools will be of the utter most importance to get a true understanding of the working mechanisms and antitumor effect of cellular therapies. The ability to track single cells, to monitor cell differentiation, tropism, migration, interactions with the environment and mechanisms of action will allow us to answer important questions regarding safety and efficacy.

To create this ideal anti-glioma agent, in this thesis we have developed several tools. First, we focused on secreted blood reporters, since they allow for sensitive, fast detection, quantification and non invasive, *ex vivo* monitoring of *in vivo* processes, with the generation of multiple data sets without the need to sacrifice the animal. Therefore, we developed an enhanced Gluc blood assay (**Chapter III**), in which Gluc is captured from the blood by an antibody-mediated reaction before bioluminescence reaction takes place. This procedure prevented signal quenching by pigmented molecules like hemoglobin, and resulted in an over one order in magnitude increase in sensitivity, allowing the detection of few circulating cells and early tumor metastases by the simple measurement of a few drops of blood.

Second, we selected an agent that is highly specific to glioma cells, while leaving healthy brain tissue unaffected. Through an extensive drug screen the Tannous lab recently discovered how the cardiac glycoside lanatoside C seems to sensitize glioma cells to anti cancer agent TRAIL. In **Chapter V** we describe how the combined treatment of sTRAIL and lanatoside C is highly effective in eradicating glioma cells and avoiding resistance and/or recurrence *in vivo*. Since TRAIL cannot cross the blood brain barrier, we decided to circumvent this problem by delivering sTRAIL directly to brain tumor environment by AAV-mediated gene delivery. By engineering the normal brain to synthesize and secrete sTRAIL, we created a zone of resistance against newly developed glioma, which can be treated with lanatoside C therapy. While treatment with sTRAIL by itself resulted in an initial decrease in tumor progression, followed by regrowth through resistant cells, no regrowth took place in the presence of lanatoside C. Further, since sTRAIL binds selectively to death receptors found only on tumor cells, no additional damage to healthy brain tissue was observed.

Third, to measure the effect of combined lanatoside C and TRAIL treatment, we developed the first triple luciferase bioluminescence imaging system for *in vivo* use (**Chapter V)**. The reporter system allowed us to sequentially monitor the changes at cellular level once therapy was initiated, with *Vargula* luciferase being used as a marker of efficient gene transfer of sTRAIL to healthy brain tissue, *Gaussia* luciferase as marker of TRAIL binding to the death receptor on Glioma cells and firefly luciferase as a marker of tumor volume and therefore therapeutic response.

Fourth, since tumorigenesis is an intricate and dynamic process and GBM cells in particular are known for their ability to escape cell death and change characteristics, we developed the first multiplex blood reporter based on secreted alkaline phosphatase (SEAP), Gaussia and Vargula luciferases (**Chapter IV**). This multiplex system differs from the triple imaging system as described in Chapter V in that here all reporters are secreted. As a result, tumor changes can be followed in real time (by simply taking an aliquot of blood), whereas the reporter from chapter V does not allow for real time imaging. Since Vargulla has not been previously used as a blood reporter we first characterized it as a secreted blood reporter. As a proof of concept we monitored the response of three different subsets of glioma cells to the chemotherapeutic agent Temozolomide (TMZ) in the same animal. U87 glioma parent cells (sensitive to TMZ) were engineered by a lentivirus vector to express Gluc (U87-Gluc), while U87R1 (resistant to TMZ) were engineered to express Vluc (U87R1-Vluc) and U87R2 (resistant to TMZ) were engineered to express both SEAP and Fluc (U87-SEAP/Fluc). All cell lines were mixed equally and intracranially implanted. One week later mice were either injected with TMZ or DMSO (control) and blood was collected at different time points. As expected, a continuous decrease in Gluc signal was observed over time in the TMZ group, while an increase in signal was observed in the control group. Both Vluc and SEAP levels increased over time in both treatment and control group, showing that U87R1 and U87R2 cells are indeed resistant to TMZ. No signal bleeding or substrate cross reaction was observed, proving that these 3 reporters can be used simultaneously in the same animal. This new multiplex reporter system can be extended and applied to many different fields for simultaneous monitoring of multiple biological parameters in real time.

II. FUTURE APPLICATION

All together, these systems create the tools to develop a highly effective therapeutic/diagnostic anti-glioma agent. Since viral therapy is known to have its limitations in terms of distribution and delivery (as discussed throughout this thesis), and stem cells intrinsically posses quite some of the characteristics needed for the ideal glioma agent (tropism, penetrating the blood brain barrier, ability to track metastasis), stem cells would be the ideal carrier for our toolbox. In this case, the combined sTRAIL/lanatoside C therapy approach could be applied to stem cell carrier system, in which stem cells will be modified to express sTRAIL. These cells can either be injected in the tumor cavity after surgery or even be given systemically. The genetically modified stem cells can be tracked using the enhanced Gluc blood assay, visualizing patterns of migration and tropism over time. Since the assay is sensitive enough to pick up signals from as little as 10 cells, the ability of the modified stem cells to invade the healthy brain parenchyma to capture single invasive glioma cells can be evaluated, as well as the specificity of the stem cells to glioma. Simultaneously, *Vargula* luciferase under the control of NFκB responsive elements, which can be detected either in the blood or by means of BLI, can be used to monitor delivery and binding of sTRAIL to the death receptors on glioma cells, while Fluc imaging (or the SEAP blood assay) can be used to evaluate the tumor response to TRAIL and lanatoside C. This approach will for the first time allow monitoring of several biological processes in response to therapy, giving new insight in the complex interactions of therapeutics to cancer cells. Obviously, other parameters can be chosen, since this system is applicable to any biological process of interest. Further, since lanatoside C, AAV vectors, stem cells and TRAIL have been previously used in the clinic separately, this combined therapeutic approach should be easily adaptive to the clinical setting.

III. CREATING THE IDEAL LUCIFERASE

Once working on optimizing BLI reporters to monitor several tumor processes simultaneously, a second issue came to mind. While *Gaussia* Luciferase possesses many favorable characteristics needed in a reporter, including high signal intensity, favorable enzyme stability and a secretion signal, and is thereby the luciferase of

choice for many BLI applications, its use for *in vivo* imaging is limited due to its emission of blue light which is absorbed by pigmented molecules such as hemoglobin and scattered by mammalian tissues. Furthermore, its flash type bioluminescence reaction, which makes the signal decrease in intensity shortly after the substrate is added, makes it unsuited for high-throughput applications such as drug screens. Therefore, in **Chapter VI,** we started a directed evolution mediated screen for Gluc mutants with more favorable characteristics, which resulted in the generation of multiple clones with higher signal intensity, glow-like kinetics (suited for high-throughput applications) and a shift in the emission towards the red region of the spectrum (suited for *in vivo* applications). Although more work needs to be done to further optimize these clones, the first step towards a red-shifted *Gaussia* Luciferase has been made. This is not only interesting for laboratory work, but in a time where optical guided surgery is on the verge of break through, a luciferase with good tissue penetration can become of tremendous value.

IV. FUTURE APPLICATIONS

Stable Gaussia

Since the 'discovery' of the alkylating agent temozolomide no real improvement has been made in the development of anti-glioma drugs. One approach to solve this problem is the screening of small molecule libraries for potential anti-glioma hits. This process is a time-consuming and costly as thousands of molecules must be screened. Gaussia luciferase is commonly used as cell viability marker as it is secreted, allowing functional analysis of drug kinetics over time by sampling of the conditioned media. As described above, its flash like luminescence, requiring each well to be injected with substrate before the read – instead of reading complete plates at a time – severely limited the amount of data that could be obtained in a certain time. The new Gluc clones with glow type BLI will be able to simplify and speed up the current process, allowing more clones to be screened and thereby increasing the chance of finding a hit. One could also imagine the benefit of using multiple luciferase reporters at the same time (e.g., the multiplex reporter systems as developed in this thesis), in order to develop a multi-parameter high throughput screening. This would provide valuable information with regards to drug working mechanisms and gene/pathway activation - thereby eventually allowing mapping of tumor response and prediction of outcome.

Red Gaussia

Optical guided surgery, which hypothetically allows for real time visualization of tumor borders, metastatic cells and nearby vulnerable structures, could potentially create a revolution in oncologic surgery. As for now, it comprises various modalities, including PET, CT, MRI and near infrared (NIR) fluorescence. While PET, MRI and CT are great diagnostic tools, they are less suitable to translate their findings to mark sharp borders of “sick” and “healthy” tissue in the operation field. Further, they are costly and not always available in OR setting. Fluorescence imaging is based on a fluorescent probe that has the ability to absorb light at a particular wavelength and subsequently emits light at a longer wavelength. Where the original fluorescent markers were not quite suitable for *in vivo* use, due to poor tissue penetration and high signal to noise ratio’s (SNR), near infra red (NIR) fluorescence (with an emission spectrum of 650-900nm) has overcome these problems. A recent pilot study in the Netherlands using the fluorescent probe fluorescein isothiocyanate (FITC) in patients with ovarian cancer was highly successful in identifying the tumor mass and metastasis < 0.5 mm, while no benign masses were colored (*Van Dam GM, Themelis G, Crane LM, et al. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: first in-human results. Nat Med. 2011;17:1315-9*). NIR fluorescence is both cost efficient and easy to use and therefore makes a good candidate for optical-guided surgery. If instead of fluorescence probes, a red-shifted *Gaussia* Luciferase could be developed, it would have the additional advantage that no external light source is needed, resulting in a further reduction of the SNR and the generation of even higher sensitivity. In animal settings, BLI is preferred over fluorescence due to this higher sensitivity. However, since *Gaussia* needs its substrate coelenterazine in order to emit light, toxicity concerns related to the substrate and immunogenicity of the *Gaussia* protein should be evaluated before the step to the clinic is made.

V. ENHANCING TISSUE CULTURE RELIABILITY

Tissue culture is the foundation for glioma research. Human glioma cell lines are differentiated, expanded, and manipulated in order to develop reliable glioma models that will increase our insight in tumor biology and give us the opportunity to test various strategies. The more reliable the tumor model, the more reliable the outcome

of the experiment and the more likely the obtained results will be reproducible in clinical setting. However, when cells are passaged over a long period of time, differentiation and phenotypic changes may occur, as well as contamination with bacteria, funghi and/or other organisms. Whereas some types of contamination are easily detected and therefore solved, others go unnoticed and severely compromise results. The latter can be said for Mycoplasma, the smallest type of free-living organism known. In order to enhance tissue culture reliability we developed a *Gaussia*-luciferase based mycoplasma detection assay, in which the rate of Gluc degeneration corresponds with the amount of Mycoplasma in the tissue culture media.

VI. FUTURE APPLICATION

Our current assay is more sensitive and cheaper than currently available commercial assays and will help researchers to work more efficient and accurate.

VII. CURRENT LIMITATIONS AND FUTURE DIRECTIVES: THE BIG PICTURE

In the beginning of this chapter two questions were raised:

- What is needed to improve treatment efficacy?
- What is needed to bridge the gap to the clinic?

Chapter-wise we discussed what actions were taken to overcome some of the limitations faced in our specific field of glioma research, in order to increase both efficacy and the likelihood of the approach being successful in clinical settings. Now it's time to look at the big picture. When looking at the glioma research field an sich one can't help but notice that very little research is able to – successfully - cross the gap between experimental setting and the clinic. Considering the amount of time (and results) that is required to 'prepare' a strategy for FDA approved clinical testing, this is remarkable.

One important limitation slowing the translation from experimental setting to clinic can be attributed to the current *glioma models*. Although many pathophysiological

similarities between the rodent glioma model and human tumors are observed, many models are based on xenografts in immunocompromised mice. Implanted tumor cells will not mimic the process of *de novo* tumorigenesis, and tumor-associated immunosuppression and immune-modulating events are not likely to be accurately reflected, resulting in a slightly different tumor microenvironment. Especially when testing immunotherapeutic approaches that require activation of the body's immune system to attack the tumor cells, this situation is les than optimal and might confound results severely. The use of rodents with an intact immune system and genetic induction of the tumorigenesis might circumvent these problems.

A second limitation arises at the level of the clinical trial itself. Since glioblastoma is a relatively 'rare' disease and since survival is poor, there's only a *limited number of patients* that can be enrolled. This results in trials with less than optimal numbers for statistical analysis. Further, since inclusion criteria differ between study groups, substantially influencing survival rates and outcome, it is hard to compare results. Also, patients often use of co-medication including corticosteroids, which impairs objective assessment even further, as efficacy of the treatment may be limited, side effects may be masked and tumor differentiation may be halted.

A third limitation is caused by difficulties related to acquiring *proper expression levels* of the to be tested therapy. Viral gene transduction levels tend to be low or decline steadily over a sufficient period of time. In both scenarios treatment efficacy is limited. This may result not only in unsuccessful clinical trials, but also in the abandonment of a potentially successful strategy. Targeting healthy brain tissue instead of the tumor itself has shown to result in more effective gene expression and might be an alternative to the current approach. In experimental setting stem- and immuno-cell carriers have shown impressive results with regards to tracking tumor cells and delivering oncolytic viruses, enzymes, cytokines, activated immune cells and other lethal substances directly to the tumor cells. However, this has yet to be demonstrated on larger scale in the clinic.

A fourth issue, closely related to the third, is appropriate *patient selection*. As discussed extensively throughout this thesis, GBM is a highly heterogeneous tumor on both molecular and genetic level. More and more evidence suggest that specific

genetic mutations in glioma cells respond to different therapies, and therefore genotyping or discovery of new biomarkers for personalized medicine could yield to an enhanced treatment success. Patient selection will not only increase patient quality of life (QoL) - withholding chemotherapy when genetic profiling will predicted the tumor is not responsive to this type of therapy - but will also give new therapeutic approaches a fair shot. Including patients with a EGFRIII mutation –which is often unresponsive to chemotherapy- for a clinical trial to test a new alkylating agent may not only potentially downplay the overall efficacy of this therapy, but may also falsely disqualify a successful approach by showing that results obtained in experimental studies cannot be repeated in the clinic.

A fifth issue is *safety.* The use of stem and/or immuno-cells, with the capacity of unlimited self-renewal, raises concerns with regards to malignant transformation. Further, the secretion of cytokines and growth factors and the local immuno-suppressive effects of stem cells could hypothetically promote tumor growth. The standard inclusion of a suicide gene in the DNA of each stem cell might help creating a safer experience. Besides safety matters, ethical issues need to be addressed. These questions do not only concern the glioma research field, but are applicable to the research community as a whole.

From the above one conclusion can be drawn: to undermine these problems and to get a true grip on a tumor as diverse as GBM a multi angle approach is needed. It is highly unlikely that – for a tumor this diverse - a single agent approach will yield the desired results. An integrated therapy based on genetic profiling that blocks several tumor escape mechanisms at once, has much more chances of succeeding.

The same can be said for the approach towards glioma research in general. A multidisciplinary, multi-angle approach is much more likely to yield results than isolated, singular attempts. To stimulate this process, the National Brain Tumor Society recently launched the DREAM Cancer Prediction Challenge, a campaign in which researchers from all backgrounds are asked to work together to attack Glioma. A virtual database, containing all available preclinical data from research groups over the world, will be generated and made accessible to participating researchers. The challenge is to design a computational model that can screen small molecule/drug

libraries against glioma, and can predict tumor response to the selected agent. If such a program can be developed, the benefit will be enormous. Not only will we be able to screen millions of compounds in a time- and cost-effective manner, it will also drastically reduce the amount of animal sacrifices needed. The data obtained by multiplex reporter systems to map the various pathways activated by certain drugs or involved in tumor escape mechanisms might play a crucial role in this project.

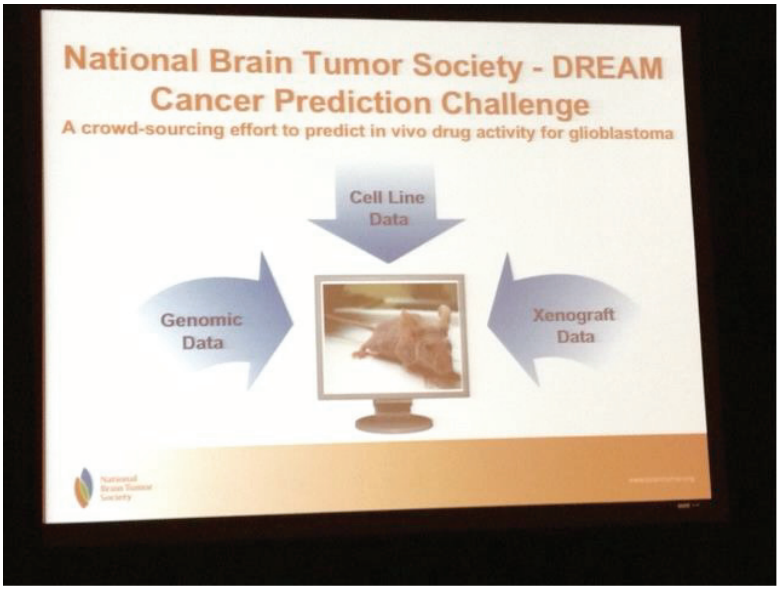

image obtained from the National Brain Tumor Society.

VII. IN CONCLUSION

In conclusion, in this thesis we have developed several tools for the road towards a better understanding of glioma biology and more effective therapy. In order to justify the importance of this work, in **Chapter XI** and **XII** we give an overview of the research being done in the neuro-oncology community and the difficulties encountered. The tools we developed (including a therapeutic agent specifically selective for glioma cells, two new multiplex imaging modalities and more sensitive assays and luciferase imaging agents) will eventually help us to translate experimental setups to the clinic, since a better understanding of the 'what' at molecular level, will result in a 'how' in a broader setting. The triple BLI and blood

assays will be of enormous value in this understanding, since they will allow us to study interactions – of tumor cells, growth factors, intrinsic pathways and therapeutics – *in vivo*; mimicking the 'real' situation as close as possible, and giving us new clues on how to increase efficacy, select new targets, prevent safety issues and eventually, to bridge the gap to the clinic.

Part 2: Discussion by Hannah Degeling

In The Netherlands, **cancer** is the leading cause of death and surpassed cardiovascular diseases in 2008. Of the 136.058 people that died in 2010, 32% of all the deaths were caused by cancer. [2] Even though great achievements are made in the battle against cancer, with a cure rate of 90% for testicular cancer as a good example, numerous cancer types are still difficult to treat:[6] patients diagnosed with glioblastoma multiforme will have a life expectancy of around 1 year. [7] The treatment of cancer remains a critical issue in our society.

The battle against cancer continues and we carry on developing new molecular imaging modalities to diagnose and monitor cancer growth. We screen for new drugs to improve chemotherapy [8], we use radio-isotopes in the field of nuclear medicine, and we even isolate the DNA gene sequence coding for the light emission in the copepod *Gaussia princeps*. We know the 'light proteins' better as luciferases and use them for bioluminescence imaging.

Bioluminescence imaging (BLI) has greatly contributed to developments in cancer research. BLI allows for monitoring of real-time tumor processes in animal models without disturbing the natural dynamics. This led to the use of BLI in xenogeneic, orthotopic, and genetically engineered animals and provided for great insight in tumor processes.[9] Even more, BLI is not only used to evaluate the characteristics of an already existing tumor, it can also provide us of more information about the onset of cancer. [10] Evidently, BLI has conquered an indispensable place in experimental research for cancer.

Nevertheless, the different luciferases used for BLI each have their disadvantages. *Gaussia* luciferase (Gluc) has a high light output, an enzymatic half-life of around 5 days, and is secreted out of the cells into the blood stream, making *ex vivo* applications possible. [11] However, Gluc has a light emission in the blue spectrum, which is, during *in vivo* imaging, mostly absorbed by pigmented molecules such as hemoglobin and is scattered by mammalian tissues before even reaching the camera. [12] Further, Gluc has a flash light emission, which can on one hand be useful for real-time monitoring, on the other hand, it makes high-throughput screening for drugs impossible. Hence, we screened for a Gluc with better characteristics in **chapter VI** and found mutants with a peak shift towards the red region of the spectrum, opening the path for further screen. The shift in wavelength is not enough

for more sensitivity during in vivo experiments. However, it does provide information about which part of Gluc is associated with the wavelength of the light emission, which will be useful for future attempts in Gluc wavelength engineering. Moreover, we found a Gluc mutant which yields stable light output without the need of a detergent such as of Triton-X 100[13], suited for high-throughput screening. High-throughput screening requires a stable signal of the luciferase in order to compare the signal of the first reading with the last one. Gluc is of great advantage for such drug-screens since it is naturally secreted out of the cells and simply measuring signal in the media is sufficient. Until now Gluc needed the addition of Triton-X 100[13] to provide a stable signal, while obviously no additional supplements to screening media are preferred. A downside of the stable mutant is its low signal intensity, making the luciferase less sensitive. Finally, we observed that all Gluc mutants contained mutations in a specific region and thereby we revealed the active site of Gluc. Once more, a better understanding of the structure of Gluc protein is very important for future Gluc engineering.

When measuring Gluc signal in the blood *ex vivo*, instead of searching for a red-spectrum Gluc, another approach to overcome Gluc signal quenching in the blood is to optimize the actual assay. In **Chapter III,** by capturing Gluc from blood using specific antibody, we developed a Gluc blood assay that showed a ten-fold higher sensitivity compared to assaying Gluc signal directly in the blood. [12] In the case of tumor, the Gluc signal in the blood can be detected at a much earlier time point and requires less tumor load. [4]

However, the antibody assay does have certain limitations. First of all the preparations and the actual assay are time consuming compared to simply assaying Gluc directly in the blood. Further, Gluc antibody has certain costs; this could be a limitation when many animals need to be monitored on a regular basis.

Nevertheless, Gluc signal in the blood is often at the low side and a more sensitive assay will be of great value. This high sensitivity will give us the opportunity to monitor very small or slow growing tumors and to detect only a few circulating tumor cells, as in the case of metastasis, which was until now difficult to achieve with Gluc. Hence, the Gluc antibody assay makes it possible to obtain more accurate information about apoptosis and the actual tumor growth. [12, 14, 15] Perhaps, this assay decreases the urgency of finding a red-light Gluc variant for *in vivo*

applications. On the other hand, the assay could also serve as a temporarily tool for more sensitivity until research found the red Gluc.

Different secreted reporters can be multiplexed together to monitor several processes in the blood at the same time *ex vivo,* given that each reporter utilizes a different substrate. [16] **Chapter IV** evaluates the potential to multiplex three different blood reporters, Gluc, secreted embryonic alkaline phosphatase (SEAP), and *Vargulla* luciferase (Vluc), and explores the possibility of a triple blood system for future research applications. Experimental research will benefit from these novel blood reporter combinations, especially when there is no need to sacrifice the animal. A downside of the reporter system is that it will take some effort to achieve a high transduction efficiency of the tumor cells and to read each time point with the three different reporters requiring three different assay procedures. .
Theoretically, an *ex vivo* blood reporter assay would have the potential for future clinical application, nonetheless, the genes coding for the blood reporters will still have to be incorporated into the DNA of our tumor cells.

As for blood reporters, research has also focused on discovering and constructing different variants for different luciferases. Not only to optimize the specific characteristics of each luciferase, but also to expand the diversity of luciferases for imaging multiple processes simultaneously. In the case of the Italian firefly luciferase (liFluc) from luciola italica, a green and a red light emission variant was characterized as described in **chapter VII**. [17] Since Fluc is due to its benefital characteristics a popular luciferase in experimental research, it will be of great use to have different variants. Nevertheless, Fluc is partly popular due to its red light emission and the green Italian variant will most likely serve as a second marker.

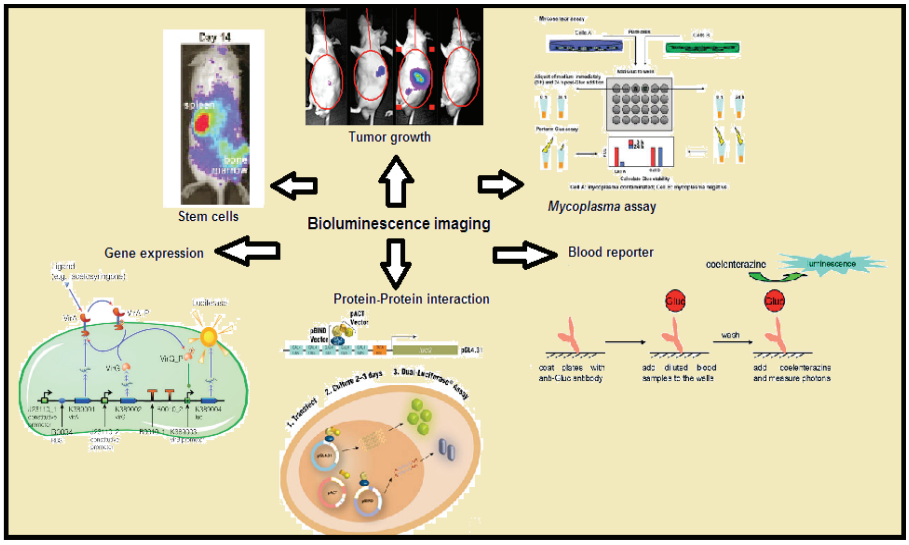

Figure 1. Bioluminescence and applications.[1,4,3,4,5]

The clinical application of luciferases is still far from being established. Even though BLI appears to be of great use in experimental animal models, a gap between these models and humans remains. What milestones should be conquered?

First of all, the human genome does not encode for the luciferases as used in experimental setting. This means that our immune system will try to eliminate the luciferase protein once expressed. Besides a potential immuno-reaction, we have not determined yet whether these foreign proteins are safe to our body, and, most importantly, what the long term effect is of expressing these foreign molecules. Secondly, luciferases require a substrate to emit light. Even if it would be safe to have cells in human expressing *Gaussia* luciferase, the necessity remains to deliver a substrate to those cells. One delivery option would be an injection into the bloodstream. However, since this substrate would bypass all our body tissues, we should be absolutely certain that no side-effects will occur.

To generate an experimental animal model orthotropic tumor cells are first transduced with the gene encoding for the luciferase, afterwards injected into the animals, and once the tumor has grown we try to eliminate it again. Obviously, in a clinical setting the tumor is already present and our main purpose is only to remove the tumor cells: the opposite principle. First of all, in contrast to experimental research, in humans we are not able to decide on the type of Glioblastoma; with a

biopsy we are merely able to observe the specific type. Secondly, when a biopsy of the tumor mass of the patient is taken and the treatment is started, we can't be sure whether the entire tumor, including the microsatellites, of the patient remains genetically identical. [18] Besides, high grade gliomas differ genetically for different individuals, limiting the possibility of finding a common molecular treatment for multiple patients. [19] [20]Thirdly, it is relatively simple to manipulate the tumor cells *in vitro* before injecting them *in vivo*, while in humans the manipulation would be performed *in vivo*, with for example viral vectors, which is much more difficult to perform successfully. Further, in public opinion, manipulation of the human genome is still controversial.

To circumvent the problems mentioned above is to use luciferases for cellular therapy against cancer, as described in the experimental part of the review of **chapter XI**. Since these stem cells are first modified *ex vivo* to carry i.a. certain drugs, theoretically it should be possible to image the migration and the production of these cells and their drugs in a clinical setting with bioluminescence. Perhaps, one day through cellular therapy, BLI can be introduced into humans.

Whereas bioluminescence imaging is nowadays restricted to experimental use, fluorescence imaging is already applied in clinical setting. Fluorescence is used for endoscopic diagnostics, for example the exploration of gastric and colonic mucosae, and can even be used during surgeries. [21] During surgery, fluorescence can provide information about the tumor edge and the distinction between normal tissue and tumor tissue. An example is 5-aminolevulinic acid, an optical dye that specifically adheres to brain tumor cells and thereby serves as a useful tool during malignant glioma resection. [22] One limitation for clinical use of fluorescence is a limited tissue penetration. Radioisotopes provide much greater depth tissue penetration. However, for surface diagnostics, such as endoscopic diagnostics, less penetration is necessary and fluorescence appears to be very useful. [22] Another limitation is the exact quantification of the signal, since different tissues absorb the light in different quantities and different tissues show different background fluorescence, resulting in a bias of the measured fluorescence signal. [21] Bioluminescence, on the contrary, is only measured when the luciferase and the substrate are actually present, which would provide more accurate results compared to fluorescence. Certainly, bioluminescence is not introduced into humans yet. Even so, with fluorescence each

dye also needs to be proven safe separately for clinical application, which definitely slows down the progress of fluorescence for clinical purposes. [21]

Another approach for the treatment of cancer, which is widely used in experimental setting and also useful for clinical purposes, is the use of **liposomes**. Liposomes provide for selective target delivery and can carry a sufficient amount of drugs. Since they are currently widely used in pharmaceutical setting, and thus approved for clinical purposes, they are of great interest. [23] An example of a successful liposomal drug is a target specific liposome containing doxorubicin to treat breast cancer metastasis. [24] However, also in the field of liposomes certain drawbacks remain, such as the quick elimination of liposomes from the blood. In the last decade, liposomes have been a popular topic in research resulting in the creation of many different variants, including long-circulating liposomes and immuno-specific liposomes. [23] One can theorize that the combination of an optimized liposome that is target specific, that can cross the blood-brain-barrier, and only releases the effective drug at the site of tumor, would be ideal for the treatment of the glioblastoma. And if we continue to speculate about future possibilities, we might even be able to incorporate the gene sequence for Gluc combined with an effective drug or an apoptotic gene sequence into a cationic liposome, and we will be able to monitor the tumor of the patient weekly by BLI.

Nonetheless, even in experimental research it would be difficult to create a liposome with all the structures mentioned above incorporated in its body. Moreover, a liposome is not like a viral vector, it doesn't incorporate into the human genome for stable expression, which means that a liposome carrying solely the gene sequence of Gluc wouldn't be of any use. The liposome would have to carry a viral vector with the Gluc sequence for Gluc expression.

Further, it would be important that the liposome has optimal MRI contrast to determine the correct location before releasing its viral vector. Still, once experimental research will have a solid and relatively simple protocol for the creation of such liposomes, they will be of great use for pre-clinical research.

Since we can find liposomes in many different shapes and formats, it is reasonable to speculate about all the different treatment possibilities with liposomes. Liposomes are not only drug carriers or DNA delivery vehicles for cancer treatment; on the contrary, liposomes are also interesting for the treatment of, for example,

neurodegenerative diseases, such as Duchenne muscular dystrophy. And liposomes have already been investigated for the delivery of a variety of agents, including nucleic acids and prednisolone, in the fight against this disease. [25] [26] Ideally in numerous of diseases, liposomes would adhere at the location of preference and locally produce our enzyme of interest. And most remarkably, research did already develop liposomes that are able to produce proteins themselves, resulting in a combined delivery and production system.[27] Therefore, what remains is the necessity of finding a perfect liposome that is very sensitive for camera detection to follow its track, it adheres specifically to the tissue of preference, and it will solely at that location release the drugs or secrete the produced protein. Conceivably, progress will be made with liposomes as protein producing vehicles and we will improve the use of essential cellular elements within a liposome.

In chapter X, we developed a liposome that is very well detectable with MRI; the liposome is target specific due to the presence of biotin at its surface and thermo-sensitive for the release of its contents. This liposome will be very suitable for the delivery of drugs to tumor cells. And hopefully, due to the possibility of tracking its faith accurately by MRI and its target specificity, this liposome will be able to serve as a promising model for a combined delivery and production system.

Many roads lead to Rome and liposomes are not the only drug delivery vehicles that can be modified for target specifity. In **chapter XI,** the use of stem cells for the treatment against brain cancer has been reviewed. Even though only one clinical trial so far is making use of these vehicles, the experimental data provide an interesting perspective. Besides serving as drug vehicles, stem cells can also deliver gene and oncolytic viral therapy at the tumor site. A prospect for experimental research would be a luciferase coupled to a virus and carried by stem cells. And theoretically, oncolytic viruses have a significant potential for glioma therapy due to their specificity and high efficiency in killing tumor cells. However, current viral therapeutic strategies have not yet reached their full potential due to poor distribution at the tumor site, low infectivity of tumor cells, and the host immune response. Therefore the strategy of hiding the virus within a stem cell has great potential. [28] Moreover, in experimental research a luciferase was coupled to TRAIL and delivered by neural stem cells. [29]

Aside from stem cells, immune cells are useful as well as described in **chapter XII**. Glioma tumor cells are clever in evading the innate immune system; immune cell

therapy can trigger an immune response instead. And since dendritic cells are potent antigen presenting cells, they emerge as promising vaccine cells in clinical trials. [30,31] For both stem and immune cell therapy, experimental research widely explores the possibilities while the clinical application advances more gradually. The translation from experimental to clinical application remains a difficult phase. The use of rodents with intact immune system, and the development of genetically-induced glioma models could help optimizing preclinical studies, leading to a more predictable transition to the clinic.

Since this thesis is about Cancer and Luciferases, the luciferases are mainly evaluated for the use in cancer research and the revelation of tumor processes. However, their value in experimental research is much broader than that and even to the extent that a disturbing *Mycoplasma* contamination can be revealed by Gluc. Gluc protein in cell culture is degraded in the presence of *Mycoplasma* and in such a stable manner that we created a Gluc Mycoplasma assay as described in **chapter VIII** and **IX**. [3]

The assay is more sensitive and less expensive than other often used *Mycoplasma* assays. A limitation is that most research doesn't make use of transduction techniques with viral vectors, and definitely not a Gluc vector, which means that Gluc media will either have to be ordered from other laboratories or a great number of cells need to be transformed with Gluc protein for a sufficient stock.

Even so, once a solid Gluc containing media stock is acquired, the assay is simple to perform and in sensitivity and expenses it is superior to a popular mycoplasma assay. [3] Therefore, it might just be a matter of time before this assay becomes the standard Mycoplasma detection assay at most cell culture laboratories.

Notably, *Mycoplasma sp.* do not only occur in cell culture; on the contrary, they are known to give for example pneumonia in humans. [32] With this knowledge, one can speculate that Gluc in clinical setting would be degraded by *Mycoplasma* as well. Even more, if *Mycoplasma* contamination has such a significant effect on Gluc, wouldn't we be able to construct a Gluc sensitive to a particular biological substance of interest? This would be of great value for future experimental research. We did speculate the possibility of this idea and constructed a Gluc protein which contains a MMP sensitive cleavage sequence. MMPs are well known biological cleavage

proteins produced by almost every cell. The constructed Gluc was expected to show a greatly reduced half-life in the presence of MMPs; however, no decrease in half-life was observed in the presence of MMPs. An explanation for this can be sought in the folding structure of Gluc, which would conceal the sensitive cleavage spot from the MMPs.

Bioluminescence and cancer: experimental perspectives

Long ago, in the nineteenth century, seamen and fishermen saw lights on the water and realized that these lights were from organisms in the water emitting them: this was called **bioluminescence**. Only about 45 years ago, various luciferases began to be characterized. [33] [34] [35] Nowadays bioluminescence is widely used in experimental research.

Novel luciferase variants are still discovered and created, which gives rise to even more imaging possibilities, such as dual/triple/quadruple imaging. Research reveals the structure of luciferases and provides thereby a manual for future luciferase engineering, resulting in optimal luciferase variants. In the last decades luciferases became important tools in experimental research.

What is it that makes luciferases valuable?

First of all, molecular cancer research needs solid tools that reveal tumor processes and monitor treatment effects. Luciferases serve as these tools; they can be attached to many different vehicles for numerous of research applications. Secondly, the variety in different kinds of luciferases creates the opportunity to use them for a wide spectrum of services; they can, for example, be used for the monitoring of gene expression, but also for tracking stem cell therapy. [29] Thirdly, certain luciferases can serve as secreted blood reporters and the *in vivo* tumor biologics can be measured *ex vivo* by these reporters. This is very useful for experimental *in vivo* research, since the animals don't need to be sacrificed for every single time point. Obviously, not all diagnostic agents require the animal to be sacrificed for the desired information and the tumor can, for example, be made visible under a camera. However, if the animals receive anesthetics before imaging it will definitely take a considerate amount of time to image all the animals for every single time point and simply withdrawing some blood will have an advantage. Besides, one of the great utilities of blood reporters is that they can provide detailed information about the

different biological processes of the tumor. Moreover, since multiple blood reporters exists, we are able to monitor the tumor response to therapy of different kinds of tumor cells at the same time, as shown in **chapter IV**. And, since blood reporters can be coupled to different genes expressed by the same tumor, our triple reporter system will make it possible to monitor noninvasively three different biological processes of the same tumor. Further, since luciferases can be expressed by all kinds of tumor cells, their value is not restricted to brain cancer research. And lastly, the lack of signal background makes them very specific compared to other imaging modalities.

Even so, luciferases could be applied in research to a much greater extent. Why the hesitation?

In the last decades molecular research has had a high focus on discovering and developing novel reporters, resulting in the availability of different reporters suited for the same purposes. Fluorescence, for example, can mark tumor cells and with the use of a laser it can easily be seen by eye. Or, for example liposomes with MRI contrast in their bilayer, they can be tracked by MRI and don't need a luciferase for monitoring. Besides, the contrast doesn't require a substrate to light up under the camera. Since current glioblastoma diagnostics and follow-up are MRI-guided, research has a relatively high focus at optimizing and developing new MRI applications. This brings us to the understanding that if bioluminescence would be introduced into humans, luciferases would not only serve as optimal tools for basic experimental research but their value would also be extended to preclinical experiments. .

Another practical limitation of bioluminescence is that a sensitive and quick reading luminometer needs to be acquired, which doesn't belong to standard laboratory equipment. However, once a luminometer is purchased it doesn't require much expertise and since the bioluminescence readings are often quick a single machine would serve for a couple of laboratories. Further, for monitoring gene expression in mammalian cells, viral vectors are needed to transduce the BLI genes, which is also not a technique every laboratory is familiar with. However, luciferases can be purchased as purified protein, they can be expressed by bacteria, and mammalian cells can easily be transformed with the protein. And even if stable transduction of the mammalian cells is required, BLI expressing cells can be provided by other laboratories or the technique of transduction can obviously be learned.

Regarding the limitations, for the expansion of luciferases in experimental research we need to continue the work of optimizing the existing variants and the creation of novel reporter systems. Further, it will be essential to have simple protocols for the usage of different BLI constructs for different applications. Luciferases do require special handling; however, with the correct equipment and techniques, they definitely have the potential of becoming valuable tools for every day laboratory use.

In conclusion, molecular research is indispensable for the understanding of cancer and luciferases play an important role in revealing these tumor processes. Therefore, in this thesis, we contributed to a better understanding of luciferases and their applicability in experimental cancer research. For (pre-) clinical purposes we expanded our field to the creation of a liposome that is susceptible for a biotin-streptavidin complex by biotin on its surface and highly sensitive for MRI. Moreover, we evaluated cellular vehicles to efficiently reach and treat the tumor target. Hopefully, in the battle against cancer, luciferases will continue to contribute to a better understanding of cancer and serve as useful guidance tools in the development of new cancer treatment.

REFERENCES

1. Hwang RF. Tumor-stromal interactions assessed by co-injection of pancreatic stellate and cancer cells. *pancreapedia*. Available at: http://www.pancreapedia.org/tools/methods/tumor-stromal-interactions-assessed-by-co-injection-of-pancreatic-stellate-and-cancer-.
2. . *Iavireport*. Available at: http://www.iavireport.org/Back-Issues/Documents/Figure 1_ Testing for gene expression in humanized mice.htm.
3. Degeling MH, Maguire CA, Bovenberg MS, Tannous BA. (2012). Sensitive assay for mycoplasma detection in mammalian cell culture. *Anal Chem.*84(9):4227-4232.
4. Bovenberg MS, Degeling MH, Tannous BA. (2012). Enhanced gaussia luciferase blood assay for monitoring of in vivo biological processes. *Anal Chem.*84(2):1189-1192.
5. Promega.
6. society Ac. (2011). Can testicular cancer be found early?
7. Freedman RA, Partridge AH. (2011). Adjuvant therapies for very young women with early stage breast cancer. *Breast.*20 Suppl 3:S146-149.
8. Badr CE, Niers JM, Tjon-Kon-Fat LA, Noske DP, Wurdinger T, Tannous BA. (2009). Real-time monitoring of nuclear factor kappaB activity in cultured cells and in animal models. *Mol Imaging.*8(5):278-290.
9. O'Neill K, Lyons SK, Gallagher WM, Curran KM, Byrne AT. (2010). Bioluminescent imaging: a critical tool in pre-clinical oncology research. *J Pathol.*220(3):317-327.
10. Badr CE, Tannous BA. (2011). Bioluminescence imaging: progress and applications. *Trends Biotechnol.*29(12):624-633.
11. Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO. (2005). Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. *Mol Ther.*11(3):435-443.
12. Wurdinger T, Badr C, Pike L, de Kleine R, Weissleder R, Breakefield XO, et al. (2008). A secreted luciferase for ex vivo monitoring of in vivo processes. *Nat Methods.*5(2):171-173.
13. Maguire CA, Deliolanis NC, Pike L, Niers JM, Tjon-Kon-Fat LA, Sena-Esteves M, et al. (2009). Gaussia luciferase variant for high-throughput functional screening applications. *Anal Chem.*81(16):7102-7106.
14. Chung E, Yamashita H, Au P, Tannous BA, Fukumura D, Jain RK. (2009). Secreted Gaussia luciferase as a biomarker for monitoring tumor progression and treatment response of systemic metastases. *PLoS One.*4(12):e8316.
15. Niers JM, Kerami M, Pike L, Lewandrowski G, Tannous BA. (2011). Multimodal in vivo imaging and blood monitoring of intrinsic and extrinsic apoptosis. *Mol Ther.*19(6):1090-1096.
16. Tannous BA, Teng J. (2011). Secreted blood reporters: insights and applications. *Biotechnol Adv.*29(6):997-1003.
17. Maguire CA, van der Mijn JC, Degeling MH, Morse D, Tannous BA. (2011). Codon-Optimized Luciola italica Luciferase Variants for Mammalian Gene Expression in Culture and In Vivo. *Mol Imaging.*

18. Haar CP, Hebbar P, Wallace GCt, Das A, Vandergrift WA, 3rd, Smith JA, et al. (2012). Drug resistance in glioblastoma: a mini review. *Neurochem Res.*37(6):1192-1200.
19. Wang Y, Jiang T. (2013). Understanding high grade glioma: molecular mechanism, therapy and comprehensive management. *Cancer Lett.*331(2):139-146.
20. Sulman EP, Guerrero M, Aldape K. (2009). Beyond grade: molecular pathology of malignant gliomas. *Semin Radiat Oncol.*19(3):142-149.
21. Taruttis A, Ntziachristos V. (2012). Translational optical imaging. *AJR Am J Roentgenol.*199(2):263-271.
22. Napp J, Mathejczyk JE, Alves F. (2011). Optical imaging in vivo with a focus on paediatric disease: technical progress, current preclinical and clinical applications and future perspectives. *Pediatr Radiol.*41(2):161-175.
23. Torchilin VP. (2005). Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov.*4(2):145-160.
24. Symon Z, Peyser A, Tzemach D, Lyass O, Sucher E, Shezen E, et al. (1999). Selective delivery of doxorubicin to patients with breast carcinoma metastases by stealth liposomes. *Cancer.*86(1):72-78.
25. Weller C, Zschuntzsch J, Makosch G, Metselaar JM, Klinker F, Klinge L, et al. (2012). Motor performance of young dystrophic mdx mice treated with long-circulating prednisolone liposomes. *J Neurosci Res.*90(5):1067-1077.
26. Negishi Y, Hamano N, Shiono H, Akiyama S, Endo-Takahashi Y, Suzuki R, et al. (2012). [The development of an ultrasound-mediated nucleic acid delivery system for treating muscular dystrophies]. *Yakugaku Zasshi.*132(12):1383-1388.
27. Murtas G, Kuruma Y, Bianchini P, Diaspro A, Luisi PL. (2007). Protein synthesis in liposomes with a minimal set of enzymes. *Biochem Biophys Res Commun.*363(1):12-17.
28. Ahmed AU, Thaci B, Alexiades NG, Han Y, Qian S, Liu F, et al. (2011). Neural stem cell-based cell carriers enhance therapeutic efficacy of an oncolytic adenovirus in an orthotopic mouse model of human glioblastoma. *Mol Ther.*19(9):1714-1726.
29. Hingtgen SD, Kasmieh R, van de Water J, Weissleder R, Shah K. (2010). A novel molecule integrating therapeutic and diagnostic activities reveals multiple aspects of stem cell-based therapy. *Stem Cells.*28(4):832-841.
30. Rodriguez A, Regnault A, Kleijmeer M, Ricciardi-Castagnoli P, Amigorena S. (1999). Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol.*1(6):362-368.
31. Kim W, Liau LM. (2010). Dendritic cell vaccines for brain tumors. *Neurosurg Clin N Am.*21(1):139-157.
32. Denny FW CWJ, Glezen WP. (1971). Mycoplasma pneumoniae disease: clinical spectrum, pathophysiology, epidemiology, and control. *J Infect Dis.*Jan(123(1)):74-92.
33. Greer LF, 3rd, Szalay AA. (2002). Imaging of light emission from the expression of luciferases in living cells and organisms: a review. *Luminescence.*17(1):43-74.
34. Johnson FH, Shimomura O. (1972). Enzymatic and nonenzymatic bioluminescence. *Photophysiology.*(7):275-334.

35. McElroy WD, Seliger HH, White EH. (1969). Mechanism of bioluminescence, chemiluminescence and enzyme function in the oxidation of firefly luciferin. *Photochem Photobiol.*10(3):153-170.