General Discussion
For a long time enucleation has been the treatment of choice for uveal melanoma. New treatment modalities have been developed e.g. transscleral thermotherapy (TTT), proton beam radiation, stereotactic radiotherapy and ruthenium application. These treatment options offer a better chance to spare the eye. Despite new treatment options, the overall survival of patients treated for uveal melanoma did not improve. Ultimately, most patients die of metastatic disease. Therefore, there is need for an effective treatment for distant metastases. Identification of genes, proteins and pathways has started to provide some insight in the dissemination of uveal melanoma. For development of novel anti-cancer strategies, more sensitive and less invasive cancer models are required, to detect and monitor tumor growth and metastatic disease in vivo.

Main objective of this thesis is the development of a new tumor model to study local tumor growth and metastatic disease and to explore tumor growth mechanisms of uveal melanoma.

Optical imaging, in particular bioluminescent reporter imaging, provides an opportunity to detect and monitor small numbers of malignant cells rapidly, in the living mouse. The high sensitivity and specificity for detection of cells in all compartments of a living animal frequently uncovers biological phenomena such as tumor escape mechanisms and patterns of tumor cell metastasis. This capability enables the study of metastatic and minimal residual disease in animal models. In the first part of the thesis (Chapter 2), a new in vivo optical imaging model for uveal melanoma is presented in which local tumor growth and metastatic disease can be studied non-invasively. For this, we have generated the OCM-1+Flp-In system. The coding sequence of the luciferase gene was integrated by targeted homologous recombination using the single FRT site in the OCM-1 cells, thus avoiding clonal variability. Inoculation of metastatic OCM-1 FRT/luciferase positive cells in the anterior chamber of the eye and induction of experimental metastases by intracardiac injection of luciferase positive uveal melanoma cells in nude mice combined with optical imaging resulted in early detection and continuous non-invasive sensitive monitoring of tumor growth and preferential sites for metastases and detailed anatomical information in vivo. These findings support the concept that organ specificity of metastases may not be solely due to differences in blood perfusion, but strongly depends on local interactions between cancer cells and the organ-specific (micro) environment. This OCM-1 FRT/luc model provides a valuable experimental setting for preclinical evaluation of the therapeutic efficacy of novel and existing agents in the same animal over time. In addition, possible responses can be easily analyzed for the different metastatic sites within the same animal.

Another advantage of this model is the opportunity to explore the functional role of different genes in the pathogenesis of uveal melanoma. For this, we have generated the OCM-1+Flp-In system (Chapter 2&3). The coding sequence of a gene of interest can be integrated by targeted homologous recombination using the single FRT site in the OCM-1 cells, thus
avoiding clonal variability. In Chapter 3 we provide novel evidence for the role of Bone Morphogenetic Protein 7 (BMP7) in uveal melanoma progression. BMP7 expression has been localized in normal epithelial tissues of the eye, particularly in different retinal cell layers and the iris. Low or non-detectable expression of BMP7 is found in the vast majority of primary human uveal melanoma tumors, while BMP7 expression is observed in normal human melanocytes. These results suggest that diminished BMP7 expression may disturb normal tissue homeostasis and contribute to malignant transformation. In our in vitro experiments, over-expression of BMP7 in OCM-1 uveal melanoma cells, using the single FRT site, resulted in significantly lower cell proliferation rate of these cells compared to the control OCM-1 FRT cells. Moreover, inoculation of OCM-1 FRT/BMP7 cells into the eye resulted in small or non-detectable in vivo tumors, compared to the control group (OCM-1 FRT). Tumors can often be viewed as deregulated developmental processes. Loss of BMP7 expression may result in a more mesenchymal invasive phenotype as indicated by enhanced vimentin expression, a marker of aggressiveness/invasiveness in uveal melanoma. In our study vimentin expression was strongly down-regulated in uveal melanoma cells over-expressing BMP7. These results suggest that re-expression of the BMP7 may result in diminished invasive growth of primary uveal melanoma. Although much remains to be understood about the complex role of BMPs signals in cancer, we demonstrate that loss of BMP7 expression during uveal melanoma progression may contribute to the acquisition of an invasive phenotype. We suggest that BMP7 may represent a novel therapeutic agent for repression of tumor growth of uveal melanoma.

In Chapter 3 we have analyzed the effect of BMP7 over-production in uveal melanoma cells for potential ‘BMP7 target genes’ in vitro. Using genearrays, we identified DAN as a target gene that was strongly induced in BMP7 over-expressing OCM-1 uveal melanoma cells, compared to control cells. This initial observation was the starting point for the presented study in Chapter 4. Diminished expression of tumor suppressor gene DAN (differential screening-selected gene aberrative in neuroblastoma also known as NO3), a Wnt/BMP antagonist, is inversely correlated to survival of uveal melanoma patients after enucleation. Our data suggest that, if DAN expression is low or absent, uveal melanoma might acquire a more aggressive and invasive phenotype. In animal models DAN exhibited BMP2 and BMP4 antagonist activity and acted as a Wnt inhibitor. The ability to inhibit tumor development and progression, in addition to its function as a tumor suppressor and its ability BMP/Wnt antagonist, underscore DAN’s importance in tumor development. The exact molecular mechanisms involved in DAN action are unclear and remain largely elusive. Further studies are, therefore, warranted to address DAN’s exact mechanism of action and involvement of TGFβ/BMP and Wnt family in uveal melanoma development, progression and metastasis.

In Chapter 5 expression of the angiogenic factors, VEGF-A, -B, -C, -D and b-FGF in uveal melanoma has been analyzed. The route by which a tumor can grow and metastasize is
determined, at least in part, by its ability to induce angiogenesis and lymphangiogenesis. The expression by tumor cells of soluble growth factors which are (lymph)angiogenic (e.g. VEGF family factors) could be an important determinant of the route of metastatic spread. We demonstrate that all tested tumorigenic uveal melanoma cell lines in vitro, experimentally induced tumors, and specimens of uveal melanoma patients expressed a wide range of angiogenic factors. These factors are known, individually or in combination, to play a direct role in uveal melanoma growth. VEGF-A, synthesized by melanoma cell lines in vitro, promoted angiogenesis, suggesting that VEGF-A may contribute to tumor angiogenesis. Protein levels of VEGF-A 165 in the aqueous humor of eyes with a uveal melanoma are correlated with patient survival, basal tumor diameter, and tumor height (Chapter 6) 17, 18. VEGF-B is expressed by a variety of benign and malignant tumors 19, 20 and VEGF-C is expressed by tumors like melanoma, breast cancer, and lymphoma 20. In a molecular profiling study 21, VEGF-C was significantly upregulated (30-fold) in highly versus poorly invasive uveal melanoma cells. In our study, VEGF-B and VEGF-C were abundantly expressed by uveal melanoma in vitro and in vivo, even though this tumor is disseminated solely by haematogenous route 22, 23. Clarigs et al. 24 showed that VEGF-C was not able to induce lymphangiogenesis in the normal eye and in uveal melanoma. However, in addition to lymphangiogenesis, VEGF-C has been found to induce the formation of new blood vessels, but only in early development and certain pathological settings including tumorigenesis 25. A few studies have reported VEGF-D in tumor tissue e.g., skin melanoma 26. We found that uveal melanoma cell lines expressed VEGF-D experimentally induced tumors and a limited number of clinical specimens. Our data support the notion that the ubiquitous expression of (lymph)angiogenic factors plays a causal role in the pathogenesis of uveal melanoma and distant metastasis. A recent study explored the therapeutic potential and mechanism of PlGF (placental growth factor), VEGF homolog, which regulates the angiogenic switch in disease but not in health. Anti-PlGF inhibited growth and metastasis of various tumors 27. Therefore, anti-angiogenesis therapy, like antibodies against PlGF, may be a new approach to the treatment of highly vascularised tumors such as uveal melanoma.

This thesis describes various concepts in current ophthalmologic fundamental research in uveal melanoma. More detailed functional studies should further establish the role of TGFβ/ BMP and Wnt signaling pathways in tumorigenesis and metastasis of uveal melanoma. Since metastatic cancer cells and neural crest cells (the origin of uveal melanocytes) share phenotypic similarities, such as high motility and invasiveness 28-30, research of specific pathways, involved in the acquisition of an invasive phenotype, is necessary. For these functional studies, the use of the FRT host cell line, OCM-1 FRT, may well be a suitable approach (figure 1). Differential target gene expression, combined with a fluorescent bioluminescent reporter for optical imaging, will provide better understanding of the molecular processes involved in tumorigenesis, dissemination and metastasis. Moreover, the animal model described in this thesis (Chapter 2) allows evaluation of new experimental treatment as exemplified by our
findings with BMP7 (Chapter 3). These studies may eventually lead to possible interventions to prevent metastatic disease of uveal melanoma in patients.

**Figure 1:** *Strategy functional studies and experimental therapeutics*

### References


22. Dithmar S, Diaz CE, Grossniklaus HE. Intraocular melanoma spread to regional lymph


