Harnessing zebrafish xenograft models for ocular melanoma treatment discovery

Yin, J.

Citation

Yin, J. (2023, December 5). *Harnessing zebrafish xenograft models for ocular melanoma treatment discovery*. Retrieved from https://hdl.handle.net/1887/3665682

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/3665682

Note: To cite this publication please use the final published version (if applicable).

Chapter 6

General Discussion and Summary

Melanoma is a type of cancer derived from melanocytes, which are the pigment-producing cells derived from neural crest progenitors that play important roles in the skin, inner ear, eye, leptomeninges, and other sites [1,2]. The most prevalent type of melanoma is cutaneous melanoma, followed by ocular melanoma, which is the most common primary intraocular malignancy in adults [3]. Despite a common melanocyte origin, cutaneous and ocular melanoma are very distinct diseases in terms of both genetic alterations driving the disease and biological behaviour, and due to the comparatively low incidence of ocular melanomas, most research to date has focused on cutaneous melanoma. This has led to a limited understanding and treatment options of ocular melanomas, a gap that we aim to address in this thesis, potentially advancing their diagnosis and treatment.

The majority of ocular melanomas (83%) originate inside the eye, and are derived from melanocytes in the uvea (named Uveal melanoma, UM) [4]. The uveal tract of the eye involved the choroid, ciliary body, and the iris [5]. In addition, ocular melanomas can also originate outside the eye, in the conjunctiva (Conjunctival melanoma, CoM). Both UM and CoM are malignancies of melanocytic origin, but they pose different pathologies, triggered by different risk factors, and require different treatment approaches. For example, although UV exposure is clearly a major risk factor for cutaneous melanoma and CoM [6], the contribution of UV exposure to UM pathogenesis is not well established [7-11]. In that sense, comprehending the different genetic traits of UM and CoM is the first step toward identifying patients at risk of metastasis and potential therapeutic targets for their systemic disease [12].

Therefore, in **Chapter 1**, we summarized the different genetic profiles of UM and CoM. UMs lack the most typical cutaneous melanoma-associated mutations such as (V-Raf murine sarcoma viral oncogene homolog B (BRAF), NRAS Proto-Oncogene, GTPase (NRAS), and Neurofibromin 1 (NF1)) and are instead characterized by a different set of genes with oncogenic or loss-of-function mutations, such as in the BRCA-Associated Protein 1 (BAP1) gene [13]. So far, BAP1 is identified as the only high penetrance gene for hereditary UM [14]. In contrast, CoM has some overlap in the genetic background with cutaneous melanoma: mutations in BRAF gene are identified in 25–35% of CoM, the vast majority of them being V600E.

Over the past decade, zebrafish have become an important model organism for studying cancer. Zebrafish models have strong translational potential in the drug development pipeline as a step in-between *in vitro* cultures and rodent studies, and small molecules observed to have disease-rescuing activity in zebrafish have made it into clinical trials. This translational potential is based on a shared homology between cell types and processes in vertebrates. This is exemplified by a recent report in which the mechanism by which $BRAF^{V600E}$ results in oncogenic competence in the progenitor neural crest cells and melanoblasts, but not in melanocytes, was based on

studies on zebrafish and human pluripotent stem cell cancer models [15,16]. Zebrafish express orthologs of 70% of human proteins and paralogs of 84% of all known disease-related genes [17]. Besides, there are a number of attributes that contribute to the rise in popularity of this organism for cancer research, namely its transparency, high fecundity, tractable genetics, and small size [18]. It is therefore not surprising that zebrafish models have been created to study melanoma at different stages, from the potential characteristics required for tumor initiation to metastasis and relapse [16,19].

In **Chapter 2** we described a new zebrafish xenograft screening platform for the rapid *in vivo* assessment of targeted therapeutics against CoM. We xenografted blood vessel reporter transgenic zebrafish with fluorescent CoM cell lines in two independent transplantation sites: an ectopic, hematogenous engraftment through the duct of Cuvier; and an orthotopic engraftment through retro-orbital injection. Based on our results, we conclude that retro-orbital and duct of Cuvier engraftment together were suitable for the recapitulation of clinical CoM: orthotopic engraftment resulted in localized primary growth, while ectopic engraftment mimicked the distant survival once CoM has metastasized. We then adapted the intravenous engraftment strategy of CoM for drug screening. We validated the system with the successful treatment of the $BRAF^{V600E}$ mutation-specific inhibitor vemurafenib against the BRAF-mutated cell line CRMM1, which did not affect the growth of the NRAS-mutated cell line CRMM2. With the generation of these models, we provided a CoM xenograft platform that allows for high-throughput screening of targeted therapies against this disease in an *in vivo* context.

While drug screens are useful tools to discover new therapeutic agents with potential high-throughput, expanding our understanding of CoM biology also allows the rational development of targeted therapies. Currently, treatment of primary conjunctival melanoma with radiotherapy, enucleation or other modalities achieves local control in more than 90% of patients [20]. However, early diagnosis is key to the success of those interventions because once metastasizing, CoM quickly adapts and evades targeted therapies [21]. Indeed, recurrence and metastasis are the main complications of ocular melanomas, and the process by which this happens is still not fully understood.

This raises the importance of understanding two crucial processes: angiogenesis and intravasation, which are sequential steps in tumor growth. Angiogenesis is vital as it provides the necessary blood supply, allowing tumors to receive oxygen and nutrients for growth. Subsequently, intravasation comes into play, enabling cancer cells to enter the bloodstream or lymphatic vessels and potentially spread throughout the body. Investigating these processes is pivotal in unraveling the mechanisms behind CoM's ability to spread and in developing strategies to target these processes for potential

therapeutic benefit. We therefore wondered how CoM tumors gain access to the blood circulation in order to metastasize.

In **Chapter 3**, we demonstrated that zebrafish xenograft CoM models can also be used to effectively study the tumor's angiogenesis potential and immune response modulation. Indeed, by xenografting cancer cells in the perivitelline space (PVS), we saw that CoM cells can induce a strong angiogenic response that attracts blood vessels. Our findings reveal that metastatic CoM cells are highly glycolytic and secrete lactate, which recruits and polarizes human and zebrafish macrophages towards an M2-like phenotype. These macrophages then elevate the levels of proangiogenic factors such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGFβ), and interleukin 10 (IL-10) in the tumor microenvironment to govern the angiogenic response towards engrafted CoM cells.

It is noteworthy that two independent metastatic CoM cell lines exhibit highly glycolytic traits that modulate tumor-associated macrophages in order to stimulate angiogenesis, as it may be a sign that this change in tumor metabolism is needed prior to the metastatic process. This observation suggests that glycolytic activity could serve as a marker for poor prognosis or as a potential avenue for novel treatments for metastatic tumors. However, it is unclear whether primary patient tumors metastasize in a manner similar to what we observed in zebrafish. To gain a better understanding, further research is needed to uncover the specific processes and pathways involved in the spread of primary CoM tumors in patients.

Our research highlights the importance of using cancer models that can recreate the complexity of physiological processes and cell types found in the environment of human tumors, and the feasibility of the zebrafish research model to study the crosstalk between melanoma and the tumor microenvironment. Indeed, we demonstrated how the signal from tumor-associated macrophages, and not from the CoM themselves, induced the angiogenic switch. In a similar way, it was recently reported how high levels of serine peptidase inhibitor, Kunitz type 1 (SPINT1) mRNA in cutaneous melanoma patients are correlated with poor prognosis and a higher tumor-associated macrophage infiltration, and that SPINT1 deficiency in the microenvironment accelerates melanoma formation via altered macrophage recruitment and activity [19,22]. Zebrafish macrophages have conserved marker gene expression and function as their mammalian counterparts [23], which suggests that the zebrafish models that we developed can be used to study the role of macrophages in the development of ocular melanomas.

Nevertheless, it is important to acknowledge that the use of established cell lines, as we used in our study, comes with its limitations, especially considering the significant heterogeneity observed in tumor mutations and cell types among any melanoma

patients. Recognizing this heterogeneity, we have explored zebrafish xenografts as a valuable tool for studying differences inherent to individual cells as we demonstrated in the previous chapters. Our goal was to determine if this model could be utilized to develop personalized patient avatars for ocular melanoma. This approach could potentially pave the way for tailored treatments closely aligned with each patient's unique condition, advancing the prospects of personalized medicine in the field of ocular melanomas.

Following this lead, in **Chapter 4**, we described the generation of zebrafish patient-derived xenografts (PDXs) to establish a robust and reliable platform for UM research and the screening of potential anti-UM drugs. We first formulated a set of methods to isolate, preserve and transiently recover viable tissues, followed by the generation of spheroid cultures derived from primary UM. All assessed tumor-derived samples formed spheroids in culture and stained positively for melanocyte-specific markers. These spheroids were labeled with fluorescence and xenografted into zebrafish through intravenous injection. Then zebrafish yielded a reproducible metastatic phenotype and recapitulated molecular features of disseminating UM. Drug treatment with navitoclax (BCL-2/BCL-xl inhibitor) and everolimus (mTORC1 inhibitor) validated the zebrafish patient-derived model as a versatile pre-clinical tool for screening anti-UM drugs and as a pre-clinical platform to predict personalized drug responses. In the future, these pipelines can be used to facilitate the implementation of personalized medicine for the ultimate benefit of cancer patients.

One of the takeaways from our zebrafish PDX study was that the combination treatment of navitoclax and everolimus significantly reduced tumor growth compared to single treatments, possibly due to the toxicity effects of those drugs at higher concentrations. Indeed, the administration of chemotherapeutic agents is usually accompanied by significant non-selective toxicity and immunosuppression. Therefore, there is a lot of interest in discovering alternative therapies against ocular melanomas devoid of such side effects as a combination or standalone treatment, including immunotherapy, and targeted therapy. In our final study for this thesis, we focused on understanding the potential effect of one of these potentially novel targeted therapies: ginsenosides.

Ginsenosides, the active compounds from ginseng extract, are promising candidates for the treatment of ocular melanoma, but the mechanism by which ginsenosides inhibit tumor growth are still unknown. In **Chapter 5**, we tested the effect of purified ginsenosides Rg3, CK, and PPD in CoM cell lines and CoM-xenografted zebrafish. We found that ginsenosides CK and PPD consistently inhibited CoM growth *in vivo* and *in vitro*. However, only in zebrafish did ginsenoside Rg3 decrease CoM burden. This discrepancy brought us to idea that maybe these compounds undergo enzymatic modification in the treated animal. Indeed, we found that engrafted CoM cells induced

inflammation, which enhanced expression and activity of Glucosylceramidase Beta 2 (Gba2) in the xenograft environment. When we inhibited or mutated Gba2, the effect of ginsenosides Rg3 and CK, but not that of PPD, was nullified. Consistently, overexpressing *GBA2* in CoM cell lines resulted in an acquired anti-tumor effect of Rg3, thus proving that GBA2 plays a key role in ginsenoside degradation. We further demonstrated that the active ginsenoside compound, PPD, induced cell apoptosis through activating PI3K/Akt and Raf/Erk pathways to exhibit an anti-CoM effect, independently of the glucocorticoid receptor. Importantly, we also found that *gba2* is activated by engraftment of breast, prostate and patient-derived UM cells.

GBA2 belongs to a group of mammalian proteins that have been identified as glucosylceramide-degrading glucosidases, enzymes that mediate the hydrolysis of glucosylceramide and cause ER stress and apoptosis of cutaneous melanoma cells [24]. Consistently, it has been reported that GBA2 expression was downregulated in human melanoma cells as compared to normal melanocytes [24]. We therefore cannot rule out a direct effect of GBA2 in CoM cells. However, our study highlights the crucial importance of the tumor environment in CoM to determine the outcome of a potential therapy, and the need for having models that resemble this complexity in cancer research. In **Chapter 2**, we demonstrate how tumor-associated macrophages, and not CoM cells, govern the angiogenic response in CoM development. In the same line, in **Chapter 5** we show how the inflamed tumor microenvironment allows a prodrug with low toxicity effects (Rg3), to be locally converted in its active form and have anti-CoM activity. This interesting observation should be further explored in the future for other glycosylated prodrugs.

In conclusion, this thesis describes the establishment of the larval zebrafish xenograft platform with conjunctival melanoma cell lines and organoids derived from patients, which opens up promising avenues for the future of melanoma treatment. Based on this approach, we have now generated models for the assessment of drug efficacy for both UM and CoM, as well as to study tumor-intrinsic biological properties, such as the angiogenic potential of different CoM lines. This not only enhances our understanding of these cancers but also holds potential for developing more effective therapies. Furthermore, we tested the effects of ginsenosides *in vitro* and in zebrafish and highlight the importance of testing potential antimelanoma drugs in model systems that recreate the tumor microenvironment found in patients. As we continue to refine and expand these models, we move closer to the realization of personalized medicine approaches for the different types of ocular melanoma, offering new hope for patients facing these challenging diseases.

References

1. van der Kooij, M.K.; Speetjens, F.M.; van der Burg, S.H.; Kapiteijn, E. Uveal Versus Cutaneous Melanoma; Same Origin, Very Distinct Tumor Types. *Cancers* **2019**, *11*, 845.
2. Gray-Schopfer, V.; Wellbrock, C.; Marais, R. Melanoma biology and new targeted therapy. *Nature* **2007**, *445*, 851-857.
3. Rietschel, P.; Panageas, K.S.; Hanlon, C.; Patel, A.; Abramson, D.H.; Chapman, P.B. Variates of survival in metastatic uveal melanoma. *J Clin Oncol* **2005**, *23*, 8076-8080.
4. McLaughlin, C.C.; Wu, X.C.; Jemal, A.; Martin, H.J.; Roche, L.M.; Chen, V.W. Incidence of noncutaneous melanomas in the US. *Cancer-Am Cancer Soc* **2005**, *103*, 1000-1007.
5. Fallico, M.; Raciti, G.; Longo, A.; Reibaldi, M.; Bonfiglio, V.; Russo, A.; Caltabiano, R.; Gattuso, G.; Falzone, L.; Avitabile, T. Current molecular and clinical insights into uveal melanoma (Review). *Int J Oncol* **2021**, *58*, 1.
6. Gandini, S.; Sera, F.; Cattaruzza, M.S.; Pasquini, P.; Picconi, O.; Boyle, P.; Melchi, C.F. Meta-analysis of risk factors for cutaneous melanoma: II. Sun exposure. *Eur J Cancer* **2005**, *41*, 45-60.
7. Chang, E.; Demirci, H.; Demirci, F.Y. Genetic Aspects of Conjunctival Melanoma: A Review. *Genes* **2023**, *14*, 1668.
8. Jager, M.J.; Shields, C.L.; Cebulla, C.M.; Abdel-Rahman, M.H.; Grossniklaus, H.E.; Stern, M.H.; Carvajal, R.D.; Belfort, R.N.; Jia, R.B.; Shields, J.A.; et al. Uveal melanoma. *Nat Rev Dis Primers* **2020**, *6*, 24.
9. Robertson, A.G.; Shih, J.; Yau, C.; Gibb, E.A.; Oba, J.; Mungall, K.L.; Hess, J.M.; Uzunangelov, V.; Walter, V.; Danilova, L.; et al. Integrative Analysis Identifies Four Molecular and Clinical Subsets in Uveal Melanoma. *Cancer Cell* **2017**, *32*, 204-220.
10. Brouwer, N.J.; Verdijk, R.M.; Heegaard, S.; Marinkovic, M.; Esmaeli, B.; Jager, M.J. Conjunctival melanoma: New insights in tumour genetics and immunology, leading to new therapeutic options. *Prog Retin Eye Res* **2022**, *86*, 100971.
11. Brouwer, N.J.; Marinkovic, M.; van Duinen, S.G.; Bleeker, J.C.; Jager, M.J.; Luyten, G.P.M. Treatment of conjunctival melanoma in a Dutch referral centre. *Brit J Ophthalmol* **2018**, *102*, 1277-1282.
12. Rodrigues, M.; de Koning, L.; Coupland, S.E.; Jochemsen, A.G.; Marais, R.; Stern, M.H.; Valente, A.; Barnhill, R.; Cassoux, N.; Evans, A.; et al. So Close, yet so Far: Discrepancies between Uveal and Other Melanomas. A Position Paper from UM Cure 2020. *Cancers* **2019**, *11*, 1032.
13. Shah, A.A.; Bourne, T.D.; Murali, R. BAP1 protein loss by immunohistochemistry: a potentially useful tool for prognostic prediction in patients with uveal melanoma. *Pathology* **2013**, *45*, 651-656.
14. Masoomian, B.; Shields, J.A.; Shields, C.L. Overview of BAP1 cancer predisposition syndrome and the relationship to uveal melanoma. *J Curr Ophthalmol* **2018**, *30*, 102-109.

15. Baggiolini, A.; Callahan, S.J.; Montal, E.; Weiss, J.M.; Trieu, T.; Tagore, M.M.; Tischfield, S.E.; Walsh, R.M.; Suresh, S.; Fan, Y.J.; et al. Developmental chromatin programs determine oncogenic competence in melanoma. *Science* **2021**, *373*, eabc1048.
16. Ribas, A.; Kefford, R.; Marshall, M.A.; Punt, C.J.A.; Haanen, J.B.; Marmol, M.; Garbe, C.; Gogas, H.; Schachter, J.; Linette, G.; et al. Phase III Randomized Clinical Trial Comparing Tremelimumab With Standard-of-Care Chemotherapy in Patients With Advanced Melanoma. *J Clin Oncol* **2013**, *31*, 616-622.
17. Osmani, N.; Goetz, J.G. Multiscale Imaging of Metastasis in Zebrafish. *Trends Cancer* **2019**, *5*, 766-778.
18. Feitsma, H.; Cuppen, E. Zebrafish as a cancer model. *Mol Cancer Res* **2008**, *6*, 685-694.
19. Frantz, W.T.; Ceol, C.J. From Tank to Treatment: Modeling Melanoma in Zebrafish. *Cells-Basel* **2020**, *9*, 1289.
20. Carvajal, R.D.; Sacco, J.J.; Jager, M.J.; Eschelman, D.J.; Bagge, R.O.; Harbour, J.W.; Chieng, N.D.; Patel, S.P.; Joshua, A.M.; Piperno-Neumann, S. Advances in the clinical management of uveal melanoma. *Nat Rev Clin Oncol* **2023**, *20*, 99-115.
21. Jiang, B.P.; Zhang, L.; Guo, X.L.; Shen, X.C.; Wang, Y.; Zhu, Y.; Liang, H. Poly(N-phenylglycine)-Based Nanoparticles as Highly Effective and Targeted Near-Infrared Photothermal Therapy/Photodynamic Therapeutic Agents for Malignant Melanoma. *Small* **2017**, *13*, 1602496.
22. Gomez-Abenza, E.; Ibanez-Molero, S.; Garcia-Morenou, D.; Fuentes, I.; Zon, L.I.; Mione, M.C.; Cayuela, M.L.; Gabellini, C.; Mulero, V. Zebrafish modeling reveals that SPINT1 regulates the aggressiveness of skin cutaneous melanoma and its crosstalk with tumor immune microenvironment. *J Exp Clin Canc Res* **2019**, *38*, 405.
23. Astin, J.W.; Keerthisinghe, P.; Du, L.; Sanderson, L.E.; Crosier, K.E.; Crosier, P.S.; Hall, C.J. Innate immune cells and bacterial infection in zebrafish. *Method Cell Biol* **2017**, *138*, 31-60.
24. Sorli, S.C.; Colié, S.; Albinet, V.; Dubrac, A.; Touriol, C.; Guilbaud, N.; Bedia, C.; Fabrias, G.; Casas, J.; Ségui, B. The nonlysosomal β-glucosidase GBA2 promotes endoplasmic reticulum stress and impairs tumorigenicity of human melanoma cells. *The FASEB Journal* **2013**, *27*, 489-498.