Modelling metastatic melanoma in zebrafish

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Chapter 7: Summary and general discussion

Cancer and melanomagenesis

Cancer is one of the leading causes of death worldwide: three out of ten disease-related deaths can be ascribed directly to the consequences of cancer[1]. Worryingly, the overall incidence of cancer is on a steady rise[1,2]. The vast majority of cancer patients do not die from a primary tumor, but instead from the metastatic spread of the disease[3]. After its dissemination from a primary tumor, cancer cells invade distant tissues and through a combination of invasion and subsequent growth perturb the functions of the organs harboring these metastatic colonies and underlying bodily functions[3–5].

One of the most common cancers found in man are melanomas. Melanomas are cancers that canonically derive from melanocytes in the skin and in the eye leading to formation of skin and ocular melanoma, respectively. Melanocytes (with the exception of uveal melanocytes) are generally thought to be transformed through subsequent mutational events induced by UV damage. The causal event driving initial oncogenic transformation of uveal melanocytes remains unknown. The predisposing factors to melanoma development are a fair skin, the inability to tan, and light eye color. The general incidence of melanoma increases with age and most patients are of Caucasian skin type. The outlook for patients with metastatic melanoma is grim, despite that, there have been considerable advances in the treatment of cutaneous melanoma (CM), including anti PD1, PDL1 immunotherapy, oncolytic virotherapy, and targeted therapy[6–8]. Ocular melanoma, such as uveal melanoma (UM) and conjunctival melanoma (CoM), are less common than cutaneous melanoma, but more lethal. This is caused by the limited availability of treatment options for these types of ocular melanoma therefore new therapeutic options are desperately needed.

Generation of ortho- and ectopic- zebrafish xenograft models for ocular melanoma.

Currently, the development of new treatments for ocular melanoma is hindered by the fact that there are few options to test potential new therapies *in vivo* prior to translation of new treatments to clinical trials. Moreover, ocular melanoma animal models generally use non-native injection sites (mainly ectopic) to mimic primary tumor formation.

In **Chapter 1**, we strove to overcome this issue through the generation of a standardized protocol for the establishment of orthotopic ocular melanoma model. This protocol generates experimental primary-like tumor through engraftment of fluorescent conjunctival melanoma cells into the retroorbital site of 48 hours post fertilization zebrafish larvae. In parallel, we created a standardized protocol to establish a disseminated, ectopic ocular melanoma model. By combining these two models, the effects of novel putative anti-cancer compounds can be assessed in an animal model at an unprecedented throughput level. We validated our methodology with the known effective therapeutic vemurafenib, which targets cancers with the BRAFV600E mutation, as found in the CoM line CRMM1. In conclusion, we established a standardized approach for the pre-clinical assessment of therapeutic efficiency in a zebrafish xenograft model with physiological and translational relevance that allows the efficacy analysis of a novel compound on a cancer cell line of interest in less than a month.

Assessment of zebrafish models for preclinical screening of conjunctival melanoma targeted therapeutics.

Although CoM is a rare subtype of ocular melanoma, its incidence worldwide is increasing. The current standard of care for primary tumors consists of surgical excision combined with secondary brachytherapy, topical chemotherapy and cryotherapy. However, CoM displays a high rate of recurrence, which associates with metastasis and overall poor prognosis. Genetically, CoM shares similar driver mutations with CM and the majority bear one of either mutually exclusive oncogenic BRAF of NRAS mutations. Currently, there are only few targeted therapies available for the treatment of CoM, namely focusing on the inhibition of oncogenic BRAF. Although there are mouse models available to study metastatic CoM, these models are limited in their applicability for drug development due to their high cost and extremely long experimental duration.

In **Chapter 2**, we developed a fluorescence-based zebrafish screening platform for the rapid *in vivo* assessment of targeted therapeutics for the treatment of CoM. To this end, we xenografted the blood vessel reporter transgenic zebrafish line Tg(*fli1*:EGFP) with conjunctival melanoma cell lines CRMM1, CRMM2 and CM2005.1 expressing lentiviral tdTomato. We investigated two commonly used sites of xenotransplantation: yolk sac engraftment, and hematogenous engraftment through the duct of Cuvier. In

addition, we developed a new orthotopic engraftment strategy, applying the newly developed method described in **Chapter 1**, that not only generates a localized primary tumor-like growth, but also enables distant metastasis at later stages.

We have validated the CoM zebrafish xenograft model using the BRAFV600E mutation specific inhibitor vemurafenib, which showed significant reduction in tumor volume for the BRAF-mutated CoM cell lines *CRMM1* and *CM2005.1*, while the NRAS mutated cell line *CRMM2* was refractory. We concluded that both retro-orbital and intravenous engraftment were suitable for the recapitulation of different stages of CoM development, where yolk sac engraftment did not yield any viable cells after implantation. We adapted the intravenous engraftment strategy of CoM for drug screening. With the generation of these models, we are the first to create and validate a CoM xenograft platform that allows for medium- to high-throughput screening of (targeted) therapies for CoM in an *in vivo* context.

Uveal melanoma zebrafish patient derived melanoma models, for the pre-clinical assessment of targeted therapy.

Despite causing up to approximately 90% of all Ocular Melanoma cases, UM is a relatively rare but deadly type of melanoma. From all patients diagnosed with UM, 50% form liver metastasis, with the liver being predominant site of metastatic dissemination for UM. After diagnosis of liver metastasis, the medium survival for UM patients is 3.9 months, independent of treatment. That is because there is currently no effective targeted therapy available for the treatment for metastatic UM. In addition, there are currently no tailored animal models that recapitulate hematogenous UM metastasis formation, and subcutaneously engrafted UM models do not spontaneously metastasize.

In **Chapter 3**, we address the issue of the limited availability of treatment options through the generation of zebrafish patient-derived xenograft (zfPDX) models for UM. We established the first reported hematogenous dissemination PDX model for UM, using the zebrafish models mentioned above. In addition, we generated a system for the cultivation of metastatic and primary UM tissue, to allow the establishment of distinct zebrafish engraftments from a single patient biopsy. During this process, we discovered that spheroid-derived, non-adherent UM samples are tumorigenic, while adherent cell lines are non-tumorigenic despite the same origin. We verified that this

phenomenon is reproducible in a set of novel adherent lines derived from tumorigenic spheroid line *spXmm66*. Upon engraftment, we confirmed that the engrafted adherent lines, independent of origin, were non-tumorigenic, whereas the spheroid line was tumorigenic. Forcing adherent lines into suspension did not provide any enhancement of tumorigenic capacity, while inhibition of ROCK signaling through ROCK inhibitor Y27632 stunted the reduction of tumorigenic capacity, possibly by ROCK mediated differentiation as reported by Maekwa et al 1999[9]. The spheroids derived from PDX derived metastatic UM tissues were used to generated a robust spheroid culture system that allowed the retention UM tumorigenic potential *in vivo*. Using these tumorigenic UM spheroid cultures, we established a zebrafish UM PDX model which was subsequently validated with previously published experimental drugs and drug combinations[10–13].

During the establishment of our zebrafish xenograft model for UM we observed the rapid clearance of circulating UM cells derived from adherent cell lines: engrafted, non-melanated, UM cells show complete clearance within 24 hours post-injection into the zebrafish host, as an exception to all other cell lines derived from other cancer types injected in our lab. We reasoned that the aforementioned attrition of circulating UM cells would be driven through a cell intrinsic mechanism, given its speed and totality. We asked if reactive oxygen species (ROS), and more specifically ferroptosis –a newly discovered mechanism of iron-dependent, ROS driven lipid oxidation-based necrosis– could be important for the observed cell death *in vivo*. We first showed that the expression of two major components, namely glutathione peroxidase 4 (GPX4) and solute carrier family 7 member 11/System Xc- (SCL7A11/XCT) involved in the maintenance and repair of ferroptosis-induced damage is inversely correlated with UM related survival. Clinical data was suggestive of a correlation between BRCA associated protein 1 (BAP1) loss, a major negative clinical prognosticator of bad disease outcome and GPX4 expression. We then assessed the efficacy of known inducers of ferroptosis in our zebrafish PDX model of metastatic UM. We leveraged the inherent advantages of our newly established model to rapidly generate patient-specific xenografts from primary UM biopsies, and used them for the assessment of ferroptosis susceptibility on a patient-to-patient basis. We induced ferroptosis through two independent pathways: via inhibition of GPX4 with RSL3, and with erastin, an inhibitor of system Xc- (SCL7A11 and SCL3A2) and mitochondrial voltage-dependent

anion channels. Both therapies significantly reduced the amount of UM cells with BAP1 loss when compared to the vehicle-treated control.

Taken together, we have provided insights in the mechanisms behind the metastatic potential of UM and, for the first time, *in vivo* preclinical evidence for the validity of ferroptosis-inducing therapy for the treatment of otherwise untreatable UM.

Modeling of metastatic melanomas in zebrafish models revealed ferroptosis as druggable pathway for clinical translation.

Melanomas are derived from melanocytes as cellular precursors and, therefore, have the intrinsic capacity to synthesize melanin. Melanin normally functions to protect the skin from free radicals generated through UV radiation[14,15] Melanin biosynthesis is driven in normal situations by the release of α-melanocyte stimulating hormone (αMSH) from the pituitary gland, where it reaches melanocytes after permeating the skin, activating the melanocortin receptors[16,17]. After binding and subsequent activation of the melanocortin receptors, Microphthalmia-associated transcription factor (MITF) translocates to the nucleus and drives expression of melanin biosynthesis via upregulating the gene expression of tyrosinase (TYR), Dopachrome tautomerase (DCT) and tyrosinase related protein 1 (TYRP1), among others[18,19].

The role of melanin in melanoma development and dissemination has been linked to a worsening of disease prognosis at an epidemiological level[20], and intracellular melanin levels have been linked to treatment resistance[21,22]. However, paradoxically, the role of melanin has also been linked to a lowered propensity to metastasize and a decrease of cellular migration[23,24].

Most melanoma cells readily produce melanin when extracted from patient tissue, but the vast majority of these cells lose melanogenic capacity *in vitro*. We have previously shown that UM lose their tumorigenic capacity when they adhere to a rigid cell culture substrate in the absence of ROCK inhibitor Y27632. In addition, in the case of *spXmm66*, we observed a co-occurrent loss of melanin in early passages. We therefore asked if the reintroduction of melanin in cells could protect UM cells in circulation, thus reinstating the lost metastatic capacity in most adherent UM cell lines. To this end we developed a co-culture system utilizing the heavily melanated cutaneous cell line MUG-Mel2 as a donor and the non-melanated UM cell lines XMM66 (adherent line derived from the same original donor as spheroid spXmm66)

and OMM2.3 as recipients. Through this coculture, we showed, for the first time, that UM cells can take in melanin from extraneous sources, and that, after melanin uptake, those cells survived significantly longer in circulation.

In **Chapter 4** we assess the observation that uveal melanoma cells derived from stable non-melanated cultures rapidly die in circulation after zebrafish xenotransplantation, as previously discussed in **Chapter 3**. In contrast we observed that, patient-derived samples, which are often intrinsically strongly melanated, survive for prolonged periods of time after hematogenous injection in zebrafish. We subsequently correlated pathological pigmentation levels at the time of enucleation with a significant reduction in disease-free survival in primary UM patients. This prompted us to ask whether the expression of melanin biosynthetic genes affect disease outcome in UM patients. Indeed, we could show that the high expression of the terminal melanin biosynthetic enzyme TYRp1 strongly correlated with a decrease in disease-free survival.

We then strove to generalize our findings to other types of melanomas. To this end, we engrafted the strongly melanated CM cell line MUG-Mel2[25] with or without prior melanin depletion. Interestingly, we found that either chemical and or genetic melanin depletion significantly reduces metastatic potential of these cells, without affecting cell viability or migratory capacity. In concordance, we demonstrated that the presence of melanin does correlate with metastatic potential in a set of tumorigenic CM and CoM cell lines.

In conclusion, we have shown that chemical perturbation of melanin biosynthesis reduces melanoma metastatic colonization in a zebrafish xenograft model across several melanoma lines and types. Moreover, we correlated TYRp1 levels with metastatic potential of melanoma lines in our zebrafish xenograft model.

Previously (**Chapter 3)**, we determined that ferroptosis was one of the main mechanisms leading to cell death of UM cells in circulation. Here, in **Chapter 4**, we demonstrated a concordant relation between the level of intracellular melanin and melanoma metastatic potential. We, therefore, hypothesized that ferroptosis might be causal to the attrition we observed in circulating UM, and melanin inhibits this process. We studied the effects of melanin on ROS, and more specifically, ferroptosis resistance of melanoma cells *in vitro*. To do so, we induced ferroptosis with RSL3 and erastin as characterized previously. We measured a significant sensitization to

ferroptosis induction in cells that were depleted of melanin prior to induction. We then validated our key findings using the zebrafish xenograft model, showing that this sensitization for ferroptosis induction through melanin-depletion also holds true in an *in vivo* context.

We further mined clinical patient data to demonstrate that most targets, if not all, of the compounds used to induce ferroptosis in our model (GPX4 for RSL3, and system Xc- components SCL7A11, SCL3A2 and VDACs for erastin), significantly negatively correlate with melanoma-specific survival in both primary CM and UM. Ultimately, we conclude that both ferroptosis resistance and melanin biosynthetic machinery are implicated in melanoma formation. These findings underscore the potential of ferroptosis-inducing strategies for the treatment of primary CM, UM, and possibly CoM patients in an adjuvant therapeutic setting.

Dissemination and standardization of zebrafish xenograft models

Since its advent in 2005, the zebrafish xenograft model is gaining in popularity[26]. Together with this rise in popularity, the need for standardization between labs also increases. Given that one of the hallmarks of the zebrafish model is its amenability to microscopic observation due to its translucent tissue architecture, many zebrafish xenograft experiments heavily utilize microscopic image-based analysis.

In **Chapter 5** we strove to combine the abundance of zebrafish micrographs and the need for both dissemination and standardization into a single web-based platform. We created the Xenograft Phenotype Interactive Repository (XePhIR). In doing so, we strive to integrate the present (superfluous) data and standardized protocols to create a visual, searchable database. Using linked, standardized metadata we aspire to help in the standardization and dissemination of our developed protocols and images in zebrafish community and beyond. XePhIR aims to advance usage of versatile zebrafish xenograft models against cancer.

Concluding remarks

This thesis describes the establishment of standardized protocols for the recapitulation of metastatic ocular melanoma in an experimental zebrafish model. Using this model, we generated platforms for the assessment of drug efficacy for both CoM and UM. We have discovered a possible reason for the loss of tumorigenic capacity of adherent UM cells, linking it to adherence, ROCK signaling and melanin loss. Utilizing a non-

adherent sphere culture system, we have been able to generate a tumorigenic PDX-derived UM animal model. This UM animal model was used for the preclinical development of ferroptosis-inducing strategies. In addition, we are the first to functionally link the presence of melanin in melanoma cells to its enhanced metastatic capacity. Ultimately, we combined all the generated protocols and knowledge to explore the function of melanin in the prevention of ferroptosis cell death in circulating melanoma cells (CM, CoM and UM). Finally, we developed a novel data sharing platform for the repurposing, standardization and enhanced dissemination of zebrafish xenograft data such as the one presented in this thesis.

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