Modelling metastatic melanoma in zebrafish

Groenewoud, A.

Chapter 1:

General introduction and thesis outline

Introduction

Cancer is one of the leading causes of disease-mediated death worldwide.[1] In almost all cases, cancer patients do not die from the primary tumor but from the metastatic form of the disease, and the subsequent perturbation of the functions of invaded tissues[2].

After the establishment of the original primary tumor, cells escape and enter into a blood or lymphatic vessel to disseminate passively through blood flow or lymphatic drainage. Cells subsequently anchor and extravasate and eventually outgrow, sometimes after years of dormancy[2,3]. Current treatment is focused either on the prevention of disease progression (in the case of primary tumors) or on the mitigation of symptoms (metastases).

Cancer, and by extension the mechanisms of metastasis, is commonly held in check through cell intrinsic (P53, cell cycle checkpoints, etc.) and extrinsic (adaptive and innate immune cells) means. Over time, the acquisition of multiple mutations (mostly preluded by a loss or inactivation of P53) leads to the escape of the cells from these safeguard mechanisms. During and after this malignant transformation, the cells continuously exchange signals with their surroundings, secreting growth factors influencing themselves and their surroundings or alternatively through direct contact-mediated interaction or indirect communication through vesicles (exosomes). Through this communication, the cancerous cells cultivate a pro-malignant environment, or cancerized field (original term coined by Slaughter et al in 1953)[4]. This environment, called the tumor microenvironment (TME), changes as the cancer progresses contributing to further tumor growth, treatment resistance, and metastatic dissemination (Figure 1).

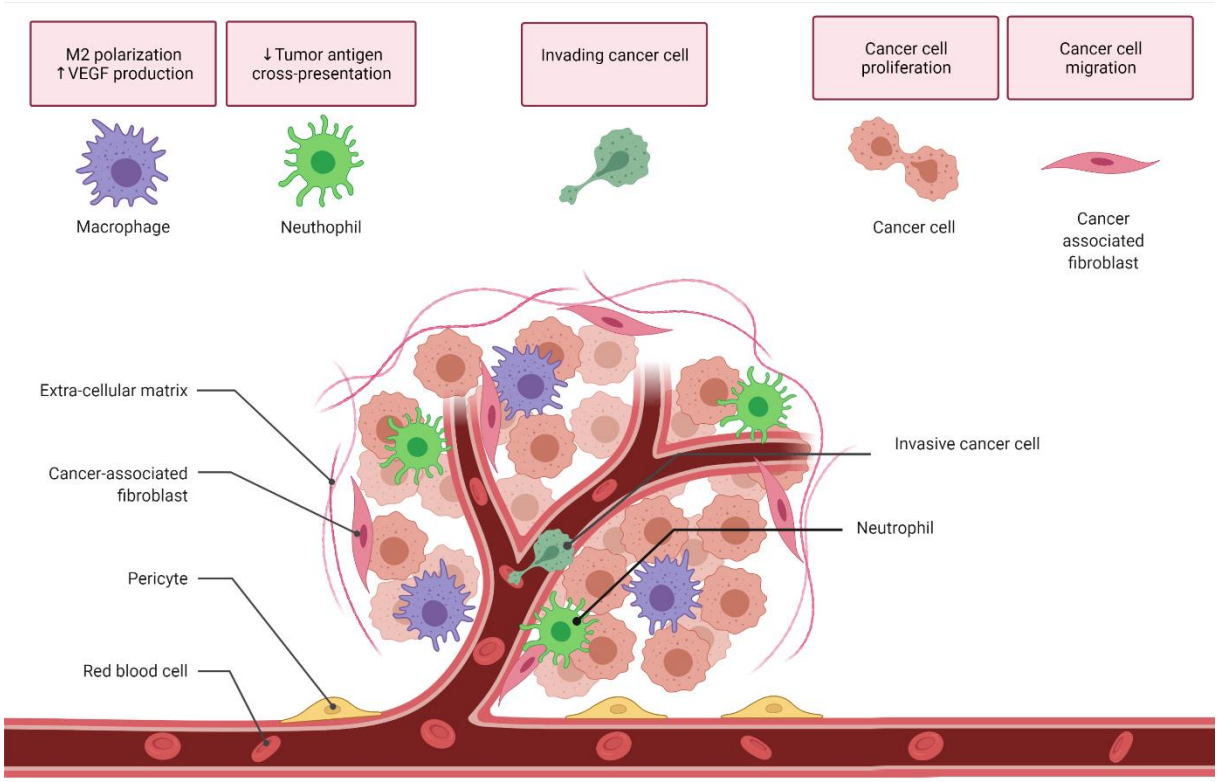

Figure 1. Cancer heterogeneity on an intertumoral level.

Primary tumors are made up of both cancer cells and "normal" non-cancer cell tissue. Intertumoral heterogeneity stems from the inclusion of these normal derived cells and extracellular matrix components, named stroma.: In many tumors a substantial percentage of the tumor volume is made up out of stroma compose of. innate and adaptive immune cells (i.e macrophages and neutrophils versus T- and NK- cells respectively), fibroblasts, extracellular matrix components and blood vessels, among others. The intertumoral heterogeneity, or the differences in clonal populations within one tumor, stems from the highly dynamic nature of the selection pressures inside the tumor. Patches of tumor subclones divide and speciate towards survival under a vast array of exogenous stimuli. Ultimately, the tumor will expand beyond the limits of its metabolic capacity, driving the recruitment of neovasculature. Cancer will then infiltrate this vasculature or invade locally into the lymphatic or vascular system and will be dispersed passively to distant sites, forming metastases. Adapted from Joyce and Pollard 2009[5].

Cancer is characterized as an uncontrolled growth of the hosts cells leading to the overgrowth and infiltration of healthy tissues, ultimately leading to metastasis to remote organs when left untreated[6,7]. During the early stages of tumorigenesis, the nascent (non-malignant, hyperplastic) cell gradually changes into a malignant (neoplastic) cell, resembling the shared etiology among malignancies[8,9]. This process is driven by the accumulation of increasing amounts of genomic mutations, thought to be preceded by a simultaneous deletion or inactivation of a tumor suppressor gene

(i.e. *P53, Rb*) and the hyperactivation of an oncogenic pathway (i.e *RAS, AKT* pathway)[10,11]. At least two mutations are needed (a loss of both alleles of a tumor suppressor gene), given that a singular instance of tumor suppressor has been shown to be insufficient for malignant transformation. This hypothesis (known as the two-hit hypothesis) explains, in part, the relative rarity of tumorigenesis on a (cellular) population basis[10]. A key part of this process is the switch of mitogenic (growth factor) dependency, to mitogenic independency, where cells shift from paracrine to autocrine mitogenic stimulation. This process further liberates the malignant cells from the control of their micro-environment.

Additional mechanisms driving tumorigenesis are acquired through sequential random somatic mutations facilitated by the cancer cell inherent genetic instability (i.e. loss of P53 or other cell cycle checkpoint proteins during tumorigenesis)[12–14]. This genetic instability greatly increases the mutational ability of cancer cells and therefore drive the microevolutionary process of cancer progression[15]. This process, combined with the increasingly hostile character of the TME, leads to the selection of mutations which allow the cell to proliferate at an increased rate, to resist cancer inhibitory immune functions, and plays an important role in the metastasis and development of resistance to treatment[5,7,16]. This gradual process enables tumor formation of high structural intricacy and heterogeneity. In the majority of tumors this increase in complexity, and concordant enhancement of stressors (nutrient deprivation, oxygen starvation etc.) within the primary tumor, some cells eventually gain metastatic capacity. This small subset of cells is not only able to disseminate but also to colonize distant organs[17].

Cancer pathogenesis and general hallmarks delineating malignant transformation

In essence, cancer cells arise from healthy cells after multiple mutations that ultimately lead to the subversion of apoptosis and the enhancement of proliferation[18]. As already mentioned, the most prominent mutations in cancer cells are mutations in tumor suppressor genes (P53, Rb etc.) and oncogenes (RAS cascade, NRAS etc.). On the one side, tumor suppressor genes mostly play a role in cell cycle progression, DNA damage repair and the integration of both processes to ensure genome stability. Inactivating mutations in such tumor suppressor genes (most commonly P53) leads to

a perturbation of the cellular safeguard mechanisms and furthers the instability of the cancer cells genome. On the other side, proto-oncogenes are genes which normally control proliferation and differentiation. These genes become oncogenic only after its mutation and subsequent enhancement of activity or effective elevation of protein levels.

In addition to the enhanced cell division capacity and a lack of programmed cell death, additional changes are required to transform a healthy cell into a cancer cell. These have been summarized as the distinct hallmarks of cancer by Hanahan and Weinberg in 2000 and updated in 2011 (Figure 2)[19]. The subsequent development of the cancer cell population is largely delineated as a small-scale evolutionary process, with selection pressure arising from the increasing hostility of the tumor micro-environment and interaction with the host immune system[20–22]. This selection pressure is thought to yield increasingly malignant cancer cells and will eventually lead to the invasion of neighboring tissues and the spreading of the cancer cells to remote organs (i.e., metastasis).

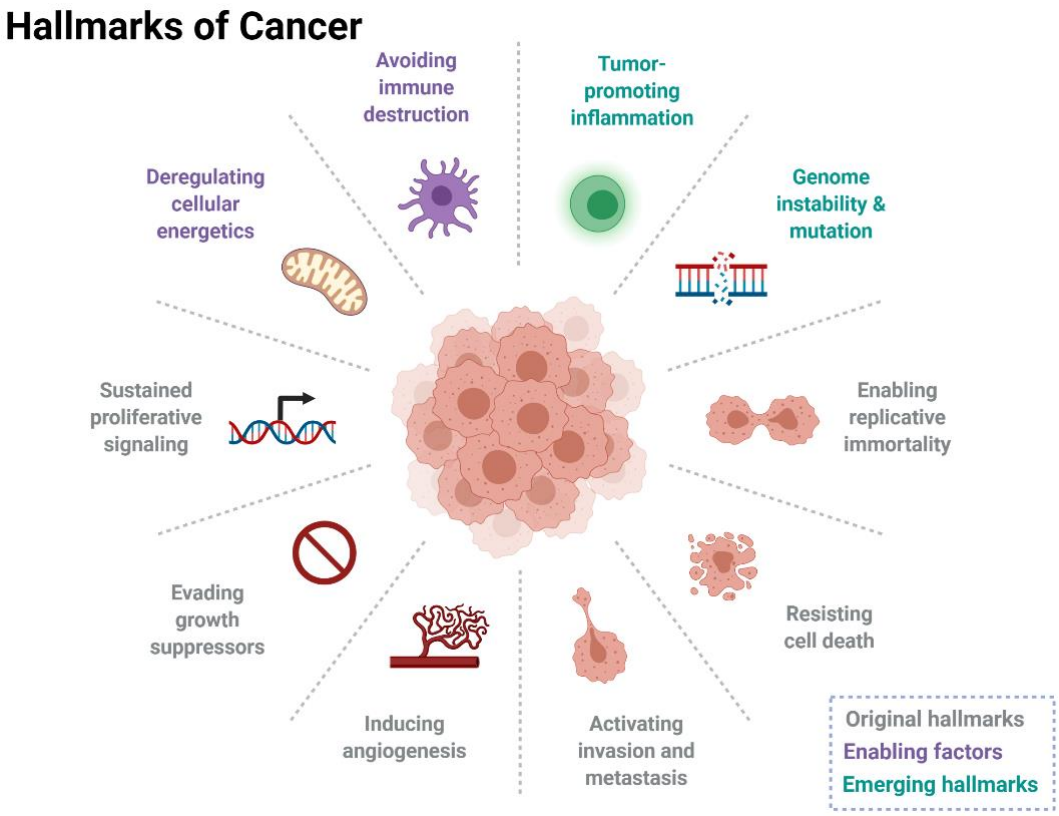

Figure 2. The canonical hallmarks of cancer.

Coined in 2000 and updated in 2011 by Hanahan and Weinberg, these features both define and drive tumorigenesis and metastasis in all cancers. Some, if not most, of these features are enhanced in tumors when compared to their wild type progenitor. Although all cancers can be seen as distinct disease entities, they have evolved mechanisms to compensate or circumvent the bodies intrinsic capacity to deal with malignant disease (evading cell death, growth suppression, immune destruction) and eventually lead to the development of metastatic capacity (inducing angiogenesis, activating invasion, migration and metastasis), which are further enhanced by cancer intrinsic mechanisms (genome instability and pro-tumorigenic inflammation). Adapted from Hannahan and Weinberg 2000&2011[18,19].

Metastasis: distant colonization, the culmination of late-stage cancer and its complications.

In 1889 Paget discovered that blood flow dictates the metastatic sites favored by metastasizing breast cancer[23]. Moreover, his findings showed that while metastatic cancer cells are found in most tissues, only in some discrete locations a metastatic colony can arise. This theory, named the “seed and soil hypothesis”, states that although cancer cells (seeds) are spread throughout the body, only in some locations where the tissue (the 'soil') is amiable to metastatic growth a metastasis will be able to sprout. Later experiments by Fiddler starting in 1970, indicated that when cells are harvested from metastatic sites they retain a certain pre-metastatic property, which can be enhanced by subsequent passages through metastatic models. These experiments hinted at a cell intrinsic mechanism that predetermines the metastatic capacity of a sub-set of cells. Moreover, the retention and amplification of these features indicated that this was presumably due to a genetic mechanism. Subsequently, Massagué and colleagues showed the existence of specific genetic drivers for metastasis in breast cancer, and that these drivers predisposed cells to grow in certain areas. With these experiments they proved that cancer cells intrinsically harbor the capacity to metastasize to all organs on a whole tumor level, but that specific sub-clones of this cancer have enhanced metastatic outgrowth capacity in common metastatic sites (brain, bone, lung and liver). Moreover, subsequent re-injection of these metastatic sub-populations demonstrated that these features can be further amplified.

To enable this passage into circulation, cancer cells have to change from their conventional stationary phenotype into a more motile and plastic phenotype, this

conversion (known as the epithelial to mesenchymal transition (EMT) in epithelial cells) allows the cells to sequentially gain cell migratory capacity while suppressing proliferation[24–27]. The signaling underlying EMT and its converse mesenchymal to epithelial transition (MET) required for the re-establishment of proliferation potential– are deemed indispensable for metastatic dissemination and outgrowth[28,29].

The canonical routes of metastasis are through the lymphatic system and the blood circulatory system, where the final steps of metastasis ultimately occur through capillary processes[30–32].Upon subsequent extravasation from circulation through either adhesion or physical entrapment in a capillary, cancer cells go through the reverse process re-establishing its epithelial phenotype and possibly generating a novel cancer cell colony, or metastasis.

Although the vast majority of all cancer patients die from the effects that the metastatic colonization has on the function of distant organs, metastasis is a highly inefficient process. This is in part explained by the previously mentioned “seed and soil hypothesis” where most of the “seeds” end up in inhospitable soil and therefore fail to grow out into a metastatic colony. This soil hostility can be seen as an oversimplification, since this context dependent tumorigenic capacity arises from both cell intrinsic mechanisms (i.e. lack of appropriate cell-cell adhesion machinery, lack of appropriate mitogen receptors) or conversely cell extrinsic (blood flow, lack of mitogen expression)[5]. Furthermore, there are a host of factors that form a functional bottleneck limiting the efficiency of metastasis (i.e. nutrient deprivation, anoikis, reactive oxygen species, immune surveillance)[7,33].

Driven by its growth and the microscale evolution underlying the developments of the primary tumor, most cancer are ultimately driven to metastasize. Metastatic dissemination can be subdivided into several different stages (Figure 3): 1) intravasation, the passage of a cancer cell form a primary tumor into a vessel (blood of lymphatic); 2) proper dissemination, the mostly passive spread of a cancer cells from a primary tumor throughout the body, a highly inefficient process thought to kill >95% of all cancer cells; 3) colonization, the adherence and survival at a distant site, eventually leading to extravasation; and 4) outgrowth, the process growing a *de novo* extravascular metastatic colony, often leading to the perturbation of organ function and ultimately host death.

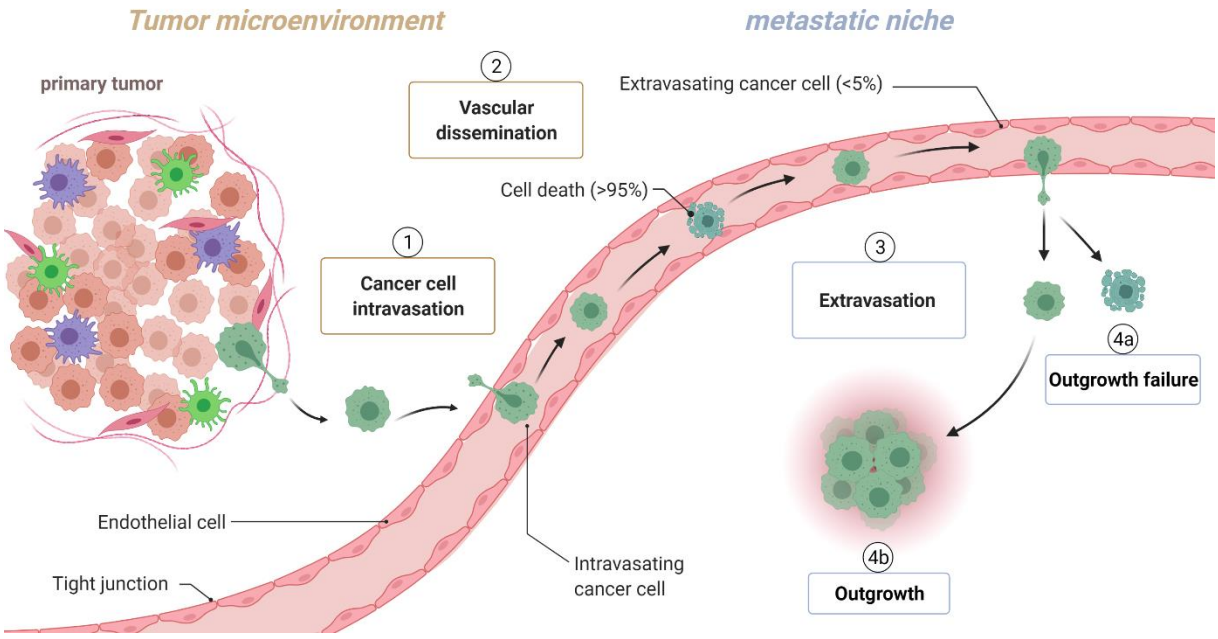

Figure 3. The process of metastasis.

Cancer cells, once intravasated, are transported through the body passively and dispersed semi-randomly following the way of least resistance, and will, in most cases, end up in either the closest lymphatic node or in the next vascularized tissue "downstream" of the tissue of origin. Next to following the physical constraint of blood flow, cancer cells will be entrapped in a capillary blood vessel and either grow out (i.e establishing a metastatic colony) or perish, either through active host interference (i.e NK-cell activation) or through a lack of viable niche (i.e., lack of required mitogens in the new environment). Despite being a highly inefficient process where 95-99% of the cancer cells do not survive, the vast majority of cancer patients (>95%) are killed by the metastatic form of the disease and not the primary tumor. Adapted from Gupta 2006 and Massague 2016[7,34].

Cutaneous, conjunctival and uveal melanoma: genetic drivers and (dis)similarities.

One of the most common types of cancer are melanomas. These cancers derive from melanocytes in the organ of origin, either in the melanocytes of the dermis, in the conjunctival melanocytes or in the melanocytes of the uvea (made up out of the iris, ciliary body and the choroid). As with all cancers, primary tumor development preludes metastatic disease formation (Figure 4).

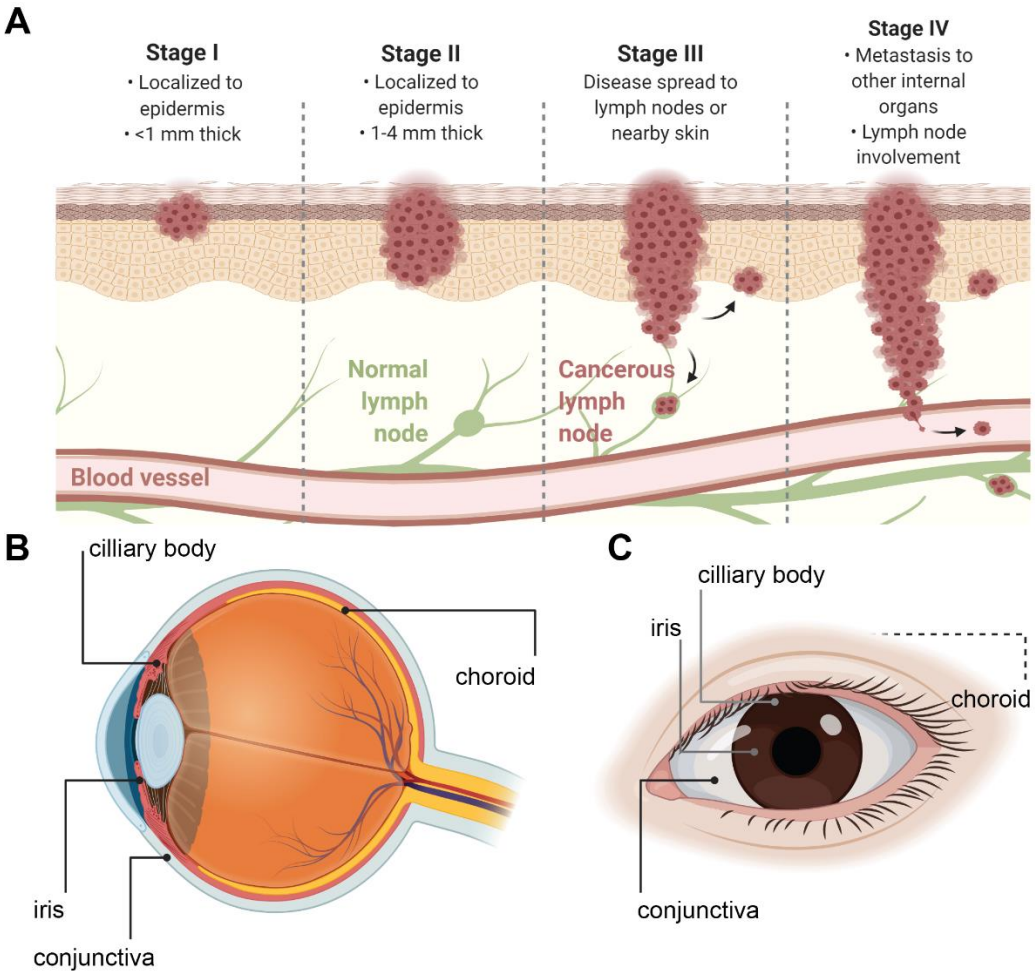

Figure 4. Melanomas and their pathological locations.

A) Cutaneous Melanoma (CM) deriving from a hyperplastic nevus (mole). As disease progresses, cells proliferate and infiltrate local tissue. Staging is largely based on size and depth of penetrance into the underlying tissue, stage I (<1 mm in thickness). Stage II, still localized to the epidermis (1-4 mm in thickness). stage III, penetration beyond the epidermis and localized micro-metastasis, cancer cells found in local lymph nodes. Stage IV (defined by lymph node involvement and metastasis to other organs). B) General location of ocular melanomas (transverse view). Uveal melanoma (UM) derives from the ciliary body, iris or choroid, whereas Conjunctival Melanoma (CoM) forms in the outer layer of the eye (conjunctiva). C) Front view of the eye, with indicated locations of UM and CoM formation. UM sites (grey lines and dotted line); note that the choroid in not visible since it is on the inside of the eye, CoM site (black line). Adapted from Damato and Coupland 2014 and Jager 2020.

Cutaneous melanoma is one of the most common malignancies in the Caucasian population, occurring in approximately 3 out of 100,000 individuals. There is high variability between populations, possibly related to the inherent skin type of the affected populations[35], moreover the overall incidence shows a steadily increasing

trend[36]. Approximately, 5 in 100,000 people are diagnosed with UM[37] and COM affects approximately 0.02 to 0.08 per 100,000 individuals per year[38].

In general, for all melanoma types**,** the prognosis for the metastatic form of the disease is grim, with an average survival of <6 months after diagnosis for metastatic CM[39], 8.1-years for metastatic CoM[38]**,** and <6 months for metastatic UM[40]. Strikingly one shared pre-disposing factor between all aforementioned melanoma is a Caucasian, light skin-type combined with blue or green eyes and an inability to tan.

Genome instability, one of the hallmarks of cancer, is one of the features that underscores the stark differences between CM, CoM and UM. Whereas CM and CoM are highly mutated, UM seems to be largely genomically stable. This genome instability subsequently drives both an enhanced risk of metastasis and an underlying basis for the development of treatment resistance. Conversely, genome instability governs the generation of neo-epitopes, used for the development of cancer immune-therapy, a highly efficient treatment option for CM, to which UM is largely refractory. Where CM and CoM cells are canonically transformed through DNA damage incurred by UV exposure, UM does not share this intrinsic UV-mediated DNA damage signature[20,41].

Ocular melanoma is relatively rare, making up approximately 3-4% of all melanomas[42,43]. Out of all ocular melanomas about 90% are uveal melanoma, with CoM making up the remaining 10%[42]. Although generally treatment of the primary tumor is effective, there is a high rate of metastasis, even as high as 50% for UM.

As previously discussed, oncogenic transformation of normal cells is conventionally driven by hyperactivation of pathways supportive of survival and proliferation, or conversely a stunting of pathways governing cell death mechanisms. One commonly implicated pathway is the MAP kinase cascade, signaling through the proteins RAS-RAF-MEK-ERK. Conjunctival melanoma share most common molecular features with CM and is, in the majority of the cases**,** driven by a hyperactivation of the RAS-RAF-MEK-ERK signaling pathway[44]**.**

Although CM, CoM and UM seem to derive from the same cell type (melanocytes), both the disease progression and therapy response is starkly different. Broadly, these cancers are grouped by their driver mutations**:** RAS/RAF for CM and CoM**,** and

GNA_q/GNA_{11} for UM (Figure 5). Oncogenic hyperactivation of these pathways or parts thereof are discussed below (Figure 5).

RAS proteins (H, K and N-RAS) are pleotropic intracellular factors that regulate pathways required for proliferation and cell survival. Dysregulation of this proto-oncogene hyperactivates these pathways and drives oncogenic transformation. RAS proteins are G-proteins possessing an intrinsic GTPase activity. The GTP-bound state is the active state and is regulated by GEFs (Guanine nucleotides exchange factors) and GAPs (GTPase activating proteins). Hyperactivating mutations in RAS proteins result in a higher fraction of the protein in the active, GTP-bound state, thereby enhancing overall RAS and downstream signaling activation. In CM, approximately 27% of all tumors carry an activating RAS mutation (HRAS (6%), KRAS (3%) and NRAS (18%)). In CoM approximately 18-19% bear a RAS mutation, with the vast majority being NRAS mutations[45]. Oncogenic mutations of RAS in UM are generally absent.[46]

In addition, activating mutations in BRAF, a signaling node immediately downstream of RAS in the MAP kinase signal transduction pathway are found in approximately 50% of CMs[47] and 30-36% of CoMs[48]. This constitutively activating mutation is generally driven by a single point mutation. Mutations of the 600th amino acid, a valine, into either glutamine (V600E) or lysine (V600K) make up the vast majority (95%) oncogenic BRAF forms[49]. As with Ras mutations, oncogenic mutations in BRAF are generally absent in UM.[50]

Signaling via PI3K-AKT-mTOR regulates cell survival through downregulation of anti-apoptotic mediators such as, for instance FOXO factors and BAD. This signal transduction pathway relies on the capacity of PI3K to phosphorylate phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2 or PIP2) generating second messenger (3,4,5)-trisphosphate (PI(3,4,5)P3 or PIP3)[51,52]. The kinases AKT and PDK1, among others, can bind to this phospholipid and are thereby recruited to the cell membrane. AKT becomes phosphorylated and activated and subsequently activate pro-survival pathways and stimulates cell growth.

Tumor suppressor gene, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) works directly to revert the conversion of PIP_2 into PIP_3 and therefore serves as a negative regulator of PI3K signaling.

Direct deregulation of PI3K by mutation is relatively common in UM but is relatively rare in CM (<3%) and is not known in CoM[53]. PTEN inactivation, in contrast, is more prevalent in most melanomas, CM (19%)[54], CoM (14%)[55] whereas in UM a loss of PTEN was reported in 16% of the assessed cases (with as much as 75% UMs showing a loss of heterozygosity)[56].

Another commonly de-regulated signal transduction pathway in cancer is the hippo pathway. One part of this pathway that is commonly hyperactivated is yes associated protein (YAP). YAP and transcriptional co-activator with PDZ-binding motif (TAZ) function as transcription factors in conjunction with interplay with TEADs, driving the expression of pro-survival genes[57]. The majority of the oncogenic functions of YAP/TAZ seem to be regulated through TEADs, although the exact underlying processes are not yet well defined. YAP/TAZ signaling is mainly implicated in the progression of UM, where it is activated through upstream Gq/G11 mutations (see figure 5)[58].

Oncogenesis of UM is largely driven through an inactivating mutation in a protein of the GNA family (GNAq and GNA11), found in approximately 90% of all cases. These mutations block the intrinsic GTPase activity within this catalytic subunit of the protein, effectively locking Gq or G11 in a constitutive active, GTP-bound state, driving oncogenic hyperactivation of Gq/G11 downstream signaling. This hyperactivation leads to a subsequent increase in downstream signaling, including the protein kinase C (PKC)/MAP kinase axis.

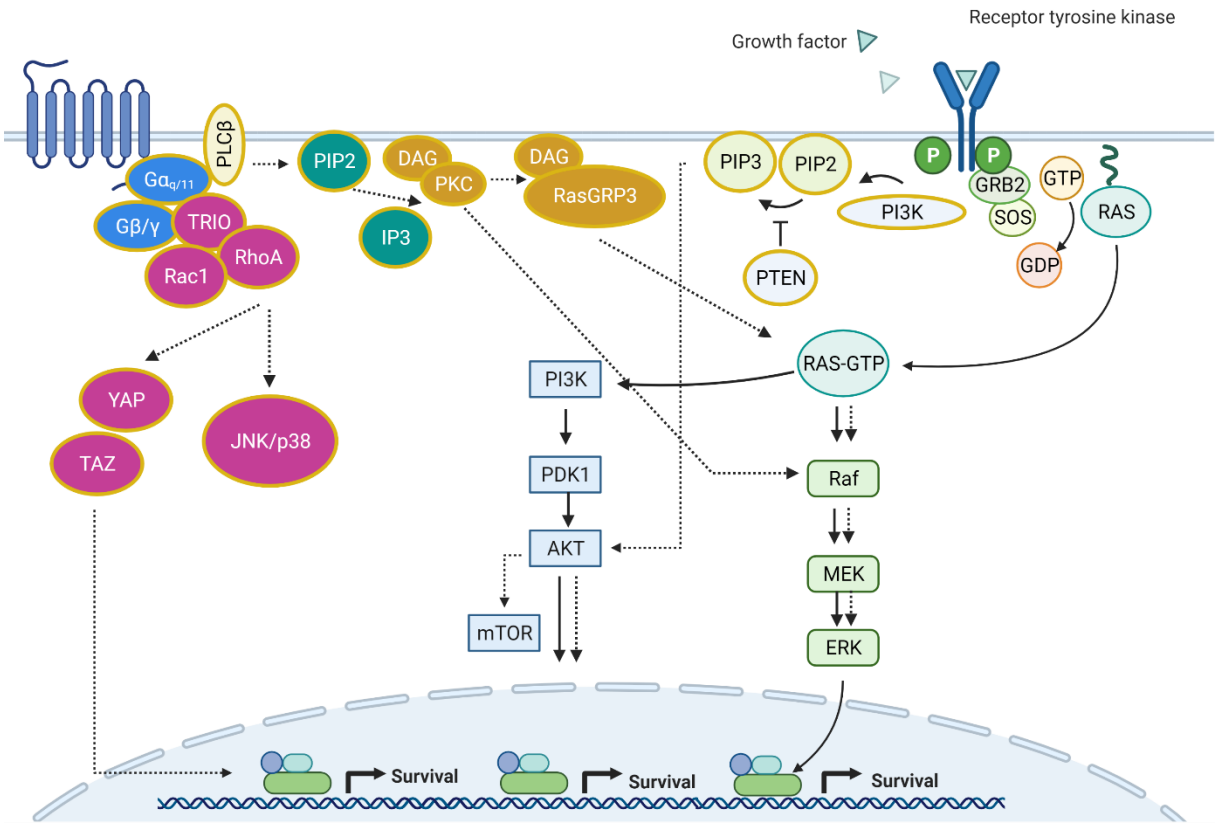

Figure 5. Similarities and differences between common driver mutations in Cutaneous Melanoma, Conjunctival Melanoma and Uveal Melanoma.

UM specific driver mutations constitutively activating $GNA_{q/11}$, upregulating phospholipase Cβ (PLCβ), protein kinase C (PKC) and GTPases RhoA and Rac. Furthermore, in UM an indirect activation of RAS along the PKC-RASGRP3-RAS axis occurs, although this non-canonical activation leads to a variegation of downstream signaling when compared to direct RAS activation. For both cutaneous melanoma (CM) and conjunctival melanoma (CoM) usually a Ras (RAS-RAF-MEK-ERK signaling axis) activation is seen as the predominant driver of oncogenesis, either through receptor tyrosine kinase hyperactivation or through direct mutational activation of RAS or downstream RAF. Either through direct or indirect activation all melanoma types (and most cancers) are dependent of downstream hyperactivation of cell survival pathways PI3K-AKT, AKT-mTOR or RAS-RAF-MEK-ERK signaling cascades. All proteins making up the signaling pathways predominantly hyperactivated in UM are bordered with orange, and the signal transduction routes are shown in dotted arrows. The proteins making up both CM and CoM are outlined in blue and signal transduction routes are shown with unbroken arrows. Adapted from Calses et al 2019, Altomare et al 2005, Chen et al 2017, Davies et al 2002 and Jager et al 2020.

Melanocyte-derived tumors, their underlying biology and melanin biosynthesis.

Cancers deriving from the melanocytes of the skin and the eye are commonly referred to as melanomas. Melanocytes are all thought to derive from a common, neuroectodermal ancestor, and after embryogenesis these cells migrate to the dermis or to the lining of the eye[59,60]. In these tissues they are believed to convey a photoprotective role through biosynthesis of melanin pigments, pheo- and eumelanin[61,62]. Generally, melanin biosynthesis is stimulated by the production of alpha melanocyte-stimulating hormone (αMSH) and its subsequent binding to the melanocortin Receptor 1 (MC1R). After ligand binding the MC1R receptor activates downstream adenyl cyclase (AC), driving up intracellular cyclic AMP (cAMP) levels. Enhanced levels of cAMP activate protein kinase alpha (PKA), which phosphorylates the transcription factor cAMP response element binding protein (CREB), which in turn enhances the transcription of the gene encoding the Microphthalmia-associated transcription factor (MITF). This transcription factor drives the expression of most melanin biosynthetic genes, and confers melanocytic identity to melanocytes (figure 6)[61,63].

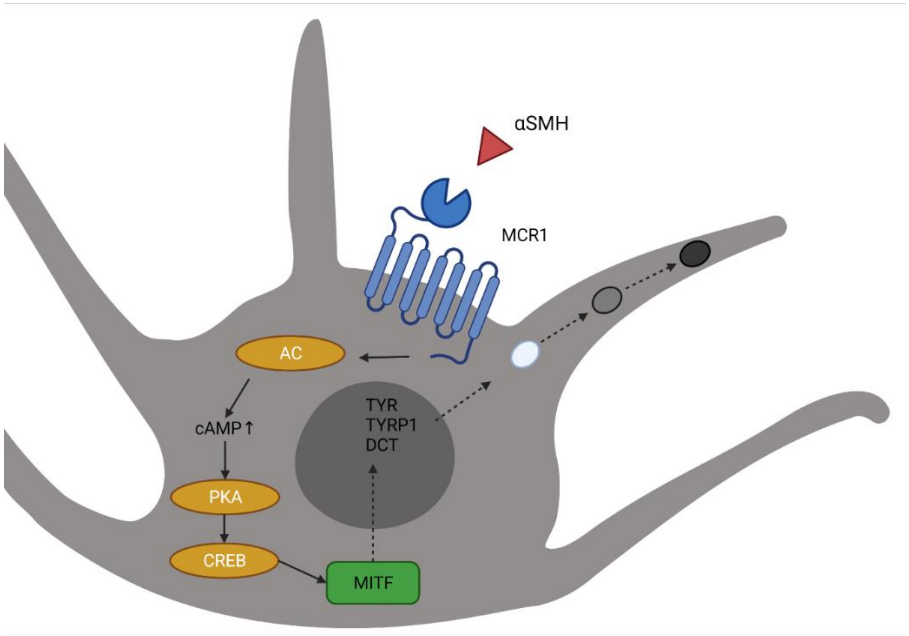

Figure 6. Melanin biosynthesis induction in melanocytes.
In untransformed melanocytes melanin biosynthesis is induced through activation of the MC1R receptor by binding of αMSH. Activation of MC1R drives intracellular activation of AC enhancing intracellular cAMP levels subsequently activation PKA and CREB, leading to MITF activation and translocation to the nucleus. MITF drives the expression of the enzymes required for melanin biosynthesis, TYR, TYRP1 and DCT. Adapted from Itoh et al 2020[64].

Melanin biosynthesis is prevalent in melanocytes as well as melanoma cells, their transformed counterpart. The widespread presence of melanin indicates a biological requirement driving the selection pressure for melanoma biosynthesis. Conversely, melanin biosynthesis is rapidly lost in *in vitro* cultures of melanoma cells. Several scientific publications attribute both anti-migratory and anti-metastatic functions to intracellular melanin[65–67].Paradoxically, within one of the previously mentioned studies, there is experimental evidence that melanin inhibits small scale migration within the primary tumor, while enhancing distant metastasis[65]. Statistical and pathological evidence indicates that higher levels of melanation result in shorter overall survival of CM patients. Taken together we conclude that the biological function of melanin in melanoma cells remains largely unknown.

Treatment options

Treatment of cancer remains highly complex, and is largely dependent on the stage of progression and the location of the specific tumor. This is further complicated by the inherent heterogeneity of tumors and the lack of highly specific markers whereupon treatment can be based[68]. Although several advances in cancer treatment have been made in the past decades, conventional treatment still largely revolves around surgical resection of the tumor, radiation- or chemotherapy, or a combination thereof[69]. With the exception of surgical resection, these treatments function through the induction of DNA damage, whereby faster dividing cells are more susceptible to damage because of its enhanced cell division. In concordance, side effects subsequently arise in untransformed, rapidly dividing tissues as the colonic mucosa or the bone marrow.

To circumvent systemic side effects newly developed drugs generally focus on the development of “personalized medicine” or “targeted chemotherapeutic” approaches as a proposed form of treatment. This approach allows focusing on the underlying molecular characterization of a tumor prior to the treatment[70,71]. For example, vemurafenib showed promising response in clinical trials for the treatment of cutaneous melanoma[72]. This therapeutic works through specific targeting of cells carrying the oncogenic, hyperactivating BRAFV600E mutation in the *BRAF* gene[73]. However, while most patients initially showed significant positive clinical response, they quickly developed vemurafenib-resistant metastases, effectively rendering this targeted therapeutic useless as a single agent treatment[74].

Given the similarities between CM and CoM on a genetic basis, and their relatively large dissimilarity with UM, we will further discuss treatment of CM and UM separately. Due to the comparatively high incidence of CM among melanomas, the largest body of experimental evidence and the most profound advances in therapeutic development have been made for CM. Generally surgical resection along with a wide margin around the affected area is employed, often combined with a sentinel lymph node biopsy to assess the possibility of system dissemination[75]. Upon diagnosis of metastatic dissemination combinations of “conventional” chemotherapeutics, targeted therapies and immunotherapies are currently used for the treatment of CM. Chemotherapeutic treatments mainly employ either DNA damage inducers dacarbazine or temozolomide. Targeted therapies against melanoma focus on RAF-MEK hyperactivation, using

either the mutation specific (BRAFV600E) inhibitor vemurafenib or possibly combined with the MEK inhibitor trametinib[47,76].

Subsequent advances in treatment of CM have come from the development of immune checkpoint inhibitors (ICI's) blocking tumor protective activities of specific T-cell ligands such as PD-1 (nivolumab and pembrolizumab) or through the blocking of CTLA-4 (ipilimumab). These antibody-based therapies focus on the activation of the host's intrinsic adaptive immune system that has been undermined during tumor development, effective re-instating host defense. These ICI's use antibodies to block the extracellular binding of either PD1 to PDL1 or CTLA4, driving the release of cytotoxic granules containing perforin and granzyme B release from the bound T-Cell, resulting in cancer cells destruction[77]. Given the overt similarities between CM and CoM many treatment options that have been proven to be clinically effective for the treatment of CM can or could be adapted for the treatment for CoM[78,79]. Strikingly, these apparent similarities in treatment response between CM and CoM do not directly translate to effective advancements in the treatment of CoM, possibly due to the low amount of clinical trials dedicated to CoM[78].

Uveal melanoma can be seen as a rare and genetically distinct subclass of melanoma and can be considered as a separate disease entity[80]. Therefore, UM is treated vastly differently from both CM and CoM. Given the discrete intra-ocular localization of this tumor the general first line treatment entails either (localized) radiation therapy or teletherapy. Alternatively complete surgical removal of the eye (enucleation), or eye-sparing treatment options are combined with radiation therapies[81–83]. Although first line treatments are generally effective, a large proportion (approximately 50%) of patients diagnosed with UM develop metastases[84]. The vast majority of UM metastasizes to the liver and there are currently no standardized treatment options for metastatic UM. Both prior treatment of primary UM and following experimental treatments of metastatic UM have not significantly enhanced patient survival[84]. All clinical trials to date that have assessed the efficacy of targeted therapies on metastatic uveal melanomas have been unsuccessful, or have been withdrawn due to intolerable side-effects[85].

Zebrafish models for metastatic melanoma and the elucidation of novel drivers of the metastatic process.

The highly complex nature of the metastatic process carries inherent difficulty to recapitulate metastasis using *in vitro* models, and in *in vivo* models the latter stages of the metastatic cascade are difficult to track. In that sense, both the semi-random nature and the difficulty to track cells during metastasis greatly limits basic research in metastatic dissemination.

The study of metastasis in murine models, generally cutaneous melanoma, has been one of the foundation stones of metastasis research. However, the use of bioluminescent imaging techniques in murine xenograft models limits spatial resolution, and yields no information of the surroundings of the metastatic colony. Both genetically engineered mouse (GEM) models and graft models (syn-, allo- and xenograft, Figure 7) have been developed for the study of metastatic spread. GEM models in general entail the overexpression and knock-down of several pro-tumorigenic factors, that eventually lead to the spontaneous formation of tumors. Although this is a highly powerful method to study the formation of primary tumors, its unpredictable nature does not make it very suitable for the study of metastatic dissemination.

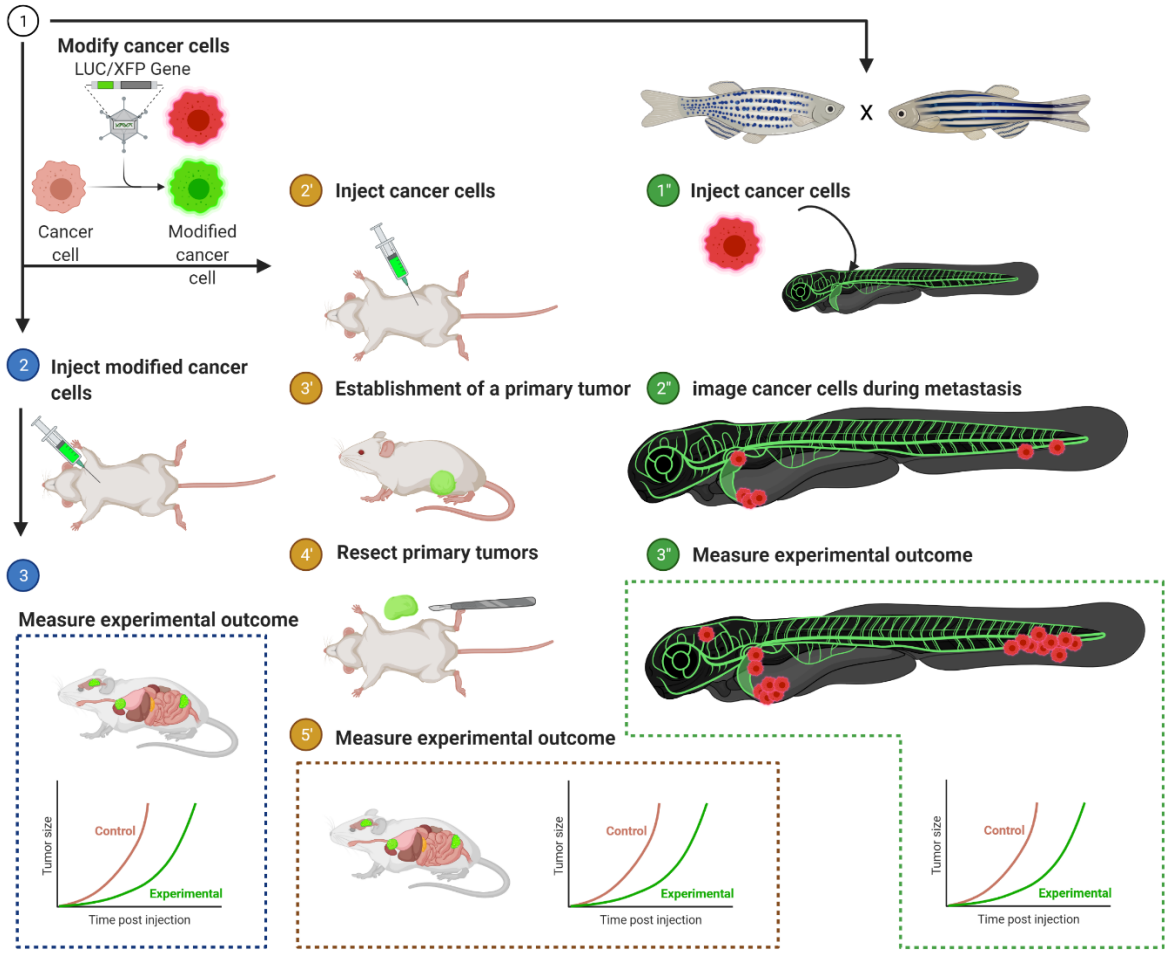

Figure 7. Schematic overview of conventional vertebrate metastasis models.

1) Modification of the engrafted cells, prior to engraftment, with either a bioluminescent reporter (Luciferase or similar proteins) and/or a fluorescent protein (XFP). Murine systemic engraftment models (delineated in blue) 1-3) intra-cardiac injection of cancer cells, allowing for quick and systemic dissemination of the engrafted cancer cells. During the time prior to the ethical endpoint of the experiment, the efficacy of cell mutations (knock-out, knock-down or overexpression) or experimental treatment efficiency can be assesed. Spontaneous metastasis models (1'-5'), utilize a similar approach but instead of directly injecting cancer cells into the blood circulation the cancer cells are first injected either sub-cutaneously, or orthotopically whenever possible. After the primary tumor reaches a pre-set diameter (before the ethical endpoint of the experiment) the primary tumor is removed surgically and the previously established spontaneous metastatic colonies are left to develop. The assessment of the effect of drugs or cell instrinsic alterations can be assessed much in a similar manner to the cardiac-injection model. Zebrafish xenografts (1"-3") allow for the injection of cells, directly into the circulation, mostly utilizing XFP based labels for the tracking of metastatic cells, in a similar manner as is commonly used for the murine cardiac-injection model.

Most of the models used for metastatic research employ fluorescent labeled cells, injected through either the tail vein or through intra-cardiac injection. This direct hematogenous injection allows for the delivery and immediate dissemination of high amounts of cancer cells, making it rapid and relatively tractable. The major downside of this method is the removal of the first stage of the metastatic cascade (intravasation) and, therefore, does not faithfully recapitulate the entire metastatic cascade. More advanced engraftment models are injected subcutaneously or orthotopically, after allowing the tumor to develop the primary growth is resected, followed by a second incubation period allowing for the establishment of distant metastatic lesions.

The zebrafish (*Danio rerio*) xenograft models as first described by Lee and colleagues in 2005 highlighted the possibility of using the zebrafish as a cancer model[86]. Since the advent of this model many variations have been proposed and rigorously assessed. Through a combination of different cancer types and injection sites we can generate discrete models for the study of primary tumor and metastasis formation. Using the zebrafish as a cancer model, we can overcome some of the challenges that hamper metastasis research. The zebrafish is hallmarked by transparent tissue architecture in its larval developmental stages. Therefore, we can use this as a model to observe the metastatic cascade from its mid- to late stages (Figure 5). Combining transgenic fluorescent zebrafish reporter lines for metastatic organs or blood vessels, we are able to closely study the complex and difficult to visualize processes of metastasis with relative ease. Moreover, the zebrafish is amenable to semi-high throughput implantation and analysis. This enables the rapid screening of compounds or the validation of the effects of genetic perturbations on the metastatic process. Taken together, the zebrafish model is an excellent platform for the study of the metastatic process and allows the tracking of metastatic cells with high spatial and temporal resolution.

In addition, the zebrafish larvae model allows for upscaling of *in vivo* analyses. Where normally a drug efficacy test *in vitro* would be performed in triplicate or quadruplicate, using zebrafish larvae we are easily capable of measuring the effect of compounds, *in vivo*, in multiples of 20 larvae per condition. In the future, development of this platform with stable, functional reporters (cell death and cell cycle reporters, etc.) integrated into the implanted cells will allow for functional readouts of the effects of drugs on implanted cells (i.e., cell cycle progression, cell death, cytoskeletal and vesicular

dynamics, etc.). Combining these models with further validation using patient derived material, generating zebrafish patient-derived xenografts (zfPDX) and experimental validation in murine models should allow cancer biologists not only to gain new insights into the biology of metastatic cancers, but also to expedite drug and therapy development through pre-screening in the zebrafish xenograft of zfPDX model.

Thesis outline

Chapter 1 provides a general introduction into cancer biology, pathogenesis and treatments, and particularly highlights cutaneous, conjunctival and uveal melanoma.

In **Chapter 2** we outline the establishment of an orthotopic zebrafish model and its use for the assessment of the efficacy of novel (targeted) cancer therapeutics. Subsequently, we give a detailed description of the methodology to generate not only metastatic tumors, but primary orthotopic eye tumors. We describe in great detail the overall methodology – starting with a novel cell line, transducing this cell line with lentiviral markers, and determining its suitability in the zebrafish xenograft model. Subsequently, we recapitulate the efficacy of a known effective inhibitor (Vemurafenib) on engrafted zebrafish, and discuss the potential pitfalls that are to be avoided while using this model.

In **Chapter 3** we use the zebrafish model for the efficacy assessment of BRAFV600E specific inhibitor Vemurafenib on conjunctival melanoma. We validate, using this novel model, the inhibitory action of Vemurafenib on conjunctival melanoma. By establishing this model, we generate a semi-high throughput screening model for the determination of drug efficacy in conjunctival melanoma, a rare cancer in dire need of an elaboration of its treatment options.

In **Chapter 4** we established patient derived spheroid cultures of uveal melanoma, the most prevalent and deadly tumor of the eye. Using the previously described zebrafish xenograft model (Chapter 2 and 3), the tumorigenic capacity of these cultures was assessed in comparison with “conventional” adherent cultures, and determined the reason underlying the loss of tumorigenic potential inherent to, most if not all, available uveal melanoma cell lines. We hypothesized that the underlying, cell autonomous, mechanism of cell death during uveal melanoma metastasis could be driven by reactive oxygen species (ROS). After analysis of patient survival databases, we

determined that high expression of ferroptosis related genes (*GPX4, SLC7A11*) significantly correlated with a decrease in disease-free survival. Subsequently we assessed the cell killing potential *in vivo* by ferroptosis induction. Ferroptosis is a recently discovered, ROS-based, iron-dependent cell death mechanism, initially shown to be effective in mutant RAS driven tumors. Experimental induction of ferroptosis was shown to be effective in reducing experimental uveal melanoma metastasis in Bap1 loss patient derived zfPDX.

In **Chapter 5** we investigate the inclusion of melanin in melanoma cells and the role that melanin plays on the negation of intracellular ROS and its effect on melanoma metastatic potential. We observed that inclusion of melanin in uveal melanoma correlates positively with engraftment rates in zebrafish xenografts. We determined, using both pathological data assigning melanin levels and transcriptional data, that the transcriptional activity of melanin biosynthesis and the overt presence of melanin significantly correlates with reduced disease-free survival in uveal melanoma patients. Subsequently we assessed the effect of melanin depletion on metastatic colonization of cutaneous melanoma in zebrafish model. We concluded that melanin depletion significantly reduces metastatic colonization, while maintaining cell migration capacity. We determined that inclusion of melanin and expression of tyrosinase related protein 1 (*TYRP1*) correlates with tumorigenic capacity in all melanocyte-derived melanomas (conjunctival, cutaneous, and uveal) in the zebrafish xenograft model. Following, we established a co-culture model to transfer melanin into non-melanated uveal melanoma and showed that re-introduction of melanin into uveal melanoma significantly enhances metastatic capacity. Finally, we showed that melanin levels increase resistance to reactive oxygen (ROS) induction both *in vitro* and *in vivo.* We showed that ferroptosis (ROS-based cell death mechanism) induction was affected *in vivo*, inversely correlating with intracellular melanin levels in conjunctival and cutaneous melanoma.

In **Chapter 6** we describe an open access, zebrafish xenograft data sharing platform for the Xenograft phenotype interactive repository (Xephir.org). This dissemination platform allows for the quick and visual determination of a xenograft suitability to a certain scientific question. Through this platform we strive to enhance visibility, accessibility, reproducibility and the overall popularity of the zebrafish xenograft model.

Finally in **Chapter 7** we summarize and discuss the preceding chapters, highlight our findings in the context of general cancer biology and provide an outlook for the implementation of our work in future research and its translation to future treatment of (uveal) melanoma patients.

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