Insights from Genetic Models of Melanoma in Fish

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Abstract Fish melanoma models are increasingly used in studies of both spontaneous and induced melanoma formation. The melanoma Xiphophorus model was the first genetic model of melanoma available to researchers since the 1920s (Klin Wochenschrift 7:1561–1562, 1928; Z Indukt Abstammungs 44:253–257, 1927). Recently, transgenesis has been used to develop zebrafish and medaka models for melanoma research (Methods Mol Biol 461:521–539, 2008). These models are now starting to produce a wealth of novel information on the genetics of melanoma (including somatic mutations), signaling pathways and molecular mechanisms, immune responses, and determinants of metastasis. Moreover, the models are extremely well suited for drug/small-molecule screens aimed at discovering new therapeutic strategies in general treatment and personalized medicine and for the study of cancer-immune cell interactions. This review provides a summary of current and prospective studies in fish models of melanoma and pinpoints the translational potentials of melanoma models in fish.

Keywords Melanoma · Cancer · Genetic models · Zebrafish · Medaka · Chemical screen

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Introduction

Melanoma is the most serious type of skin cancer, the most aggressive and lethal, affecting more than 75,000 annually in the US [4]. These malignant tumors originate from melanocytes, the pigment-producing cells of vertebrates. According to a classical model [5], melanoma begins with UV light-induced mutations activating oncogenes, which lead to melanocyte proliferation. The first response of melanocytes to oncogenes, following proliferation, is oncogene-induced senescence (OIS), which leads to the formation of a benign nevus. Upon secondary mutations inactivating tumor suppressors, OIS is overcome, and the nascent melanoma proliferates first radially in the epidermis and then vertically until cells acquire the ability to penetrate the basement membrane and further invade the dermis. In the latter stage, melanoma cells acquire metastatic characteristics and enter the blood stream or lymphatic vessels, colonizing distant tissues and organs [5]. However, not all cutaneous melanomas originate from dysplastic nevi; about 50 % occur in normal skin, which leads to the suggestion that alternative paths, including origin from melanocytic progenitor or stem cells, different transforming events or molecular pathways, are responsible for half of the cutaneous melanoma developing in humans.

The Genetic Causes of Melanoma and Fish Models

Classical studies (candidate gene sequencing, i.e., COSMIC [6]) have revealed that in over 90 % of cutaneous melanoma, somatic mutations of a handful of genes taking place in melanocytes as a result of environmental DNA damage (UV light) are at the origin of melanoma development (reviewed in [7]). These include activating mutations in oncogenes

Table 1 Transgenic fish models of melanoma

Oncogene	Promoter	Background	Onset (months)	Reference
B-RAF ^{V600E}	<i>mitfa</i>	p53 ^{−/−}	4	[4]
N-RAS ^{Q61K}	<i>mitfa</i>	p53 ^{−/−}	4	[5]
H-RAS ^{G12V}	<i>mitfa</i>		6	[6]
H-RAS ^{G12V}	<i>kita</i>		1	[9]
Xmrk	<i>mitf</i>		1	[11]

The models based on the oncogenes B-RAF^{V600E}, N-RAS^{Q61K} and H-RAS^{G12V} are zebrafish models. The model based on Xmrk is a medaka model

(including *B-RAF* 45 %, *N-RAS* 13–25 % and *H-RAS* 1–5 %) and loss of function mutations in tumor suppressors (*CDKN2A*, *RBI*, *PTEN*, *P53*). Already from these classical studies it was clear that most oncogene-activating mutations require additional factors to lead to melanoma development.

Fish melanoma models based on this information were generated (Table 1), and their studies confirmed the requirement for cooperating mutations (p53 loss for B-RAF^{V600E} [8] and N-RAS^{Q61K} [9]; PI3K activation in H-RAS^{G12V} [10]) at least when the driver oncogene was expressed under the *mitfa* promoter, a promoter driving expression in melanocytes or committed melanoblasts [11]. Intriguingly, H-RAS^{G12V} was shown not to require p53 or PTEN inactivation, and to give rise to melanoma in fish, whether its expression was driven by *mitfa* or *kita* (expressed at higher levels in melanocytes or committed melanoblasts [12]) promoters with the only difference of a much shorter latency for *kita*-driven H-RAS^{G12V} melanoma development [13]. Similarly, the Xmrk oncogene (a constitutively active form of the EGF receptor, isolated from a spontaneous melanoma model in *Xiphophorus* [14]) was shown not to require any cooperating mutations in a medaka model [15] when expressed under the *mitf* promoter. Levels and persistence of oncogene expression [13] and strength of downstream target activation [15] were considered important factors in melanomagenesis, although in the medaka model the genetic background of the fish was clearly able to influence the type of melanoma (exophytic, i.e., developing externally, uveal or invasive melanoma), which ultimately developed.

The application of whole exome and whole genome sequencing to a large number of primary and metastatic melanomas (TCGA, <http://cancergenome.nih.gov/>), while confirming the UV signature (substitution of thymine with cytosine, C > T) and the targets (*B-RAF*, *N-RAS*, *H-RAS*) also revealed a number of novel driver mutations that appeared with remarkably high frequency in melanoma, thus providing insights into the cooperating mutations. These are: *PREX2* (phosphatidylinositol-3,4,5-triphosphate-dependent Rac exchange factor 2), a negative PTEN

regulating factor [16], *PPP6C* (a phosphatase subunit regulating Aurora A kinase [17], *STK19* (a predicted kinase with unknown function) and especially *RAC1* (a rho GTPase, which regulates cytoskeleton rearrangements [18]. Moreover, mutations in the promoter region of the *TERT* gene (coding for the catalytic subunit of telomerase), leading to the generation of binding sites for the widely expressed ETS transcription factors, were reported in 50–70 % of all melanomas [19].

These studies provide the inside information for the design of new melanoma models necessary for in-depth understanding of the molecular pathogenesis and disease development in complex organisms, as highlighted in the next paragraph.

Novel Pathways in Oncogenesis Discovered or Confirmed Through the Fish Models

Since the first tumor modeled in zebrafish in 2003 [20], many studies have clearly emphasized the powerful strategy of using fish to dissect the molecular mechanisms underlying human malignancies.

In the melanoma field, many discoveries of novel pathways dysregulated in this cancer type came from the zebrafish models, starting with that generated by Patton et al. [8], where a zebrafish transgenic line expressing human activated B-RAF^{V600E} under the melanocyte-specific *mitfa* promoter developed nevi and malignant tumors in a p53-deficient background. This zebrafish melanoma model was the starting point for many important discoveries, and further insights came from the models generated by the Hurlstone [10] and Schart's groups [15], and by our laboratory [13].

Epigenetic Changes in Melanoma Initiation and Progression

Zebrafish has been used as a platform for cancer discovery thanks to the creation of a rapid screen using an innovative transgenic strategy, called miniCoopR, where candidate melanoma modifiers can be tested for their ability to initiate or accelerate melanoma (see [21•] for a description of the strategy). Through the generation and analysis of more than 3,000 transgenic animals, SETDB1, an enzyme that methylates histone H3 on lysine 9 (H3K9), was found to accelerate melanoma formation in zebrafish [22•]. Interestingly, this gene has been recently identified as significantly mutated in cancer in a massive analysis of somatic point mutations in exome sequences from almost 5,000 human cancer samples across 21 cancer types [23].

Global hypomethylation of the melanoma genome has been reported [24] and is also a hallmark in zebrafish

melanoma [25]. More recently, loss of 5-hydroxymethylcytosines (5-hmC) was identified as a discriminant between nevi and malignant melanoma [26]. In the same study, the miniCoopR vector was used to overexpress isocitrate dehydrogenase 2 in a fish B-RAF^{V600E}/p53-melanoma model shown to promote an increase of 5-hmC levels and to prolong tumor-free survival of transgenic fish.

The importance of epigenetic changes in the initial transformation and progression of melanoma emerges from results obtained in our laboratory [25]. We used the *kita*:H-RAS^{G12V} melanoma model [13] to show that oncogenic RAS is able to increase the levels of three microRNAs at the onset of melanocyte transformation (miR-146a, miR-146b and miR-193a). Interestingly, these three microRNAs have as common target, Jmjd6, a jumonjiC domain protein that functions as histone arginine demethylase [27] and mRNA splicing regulator [28]. The upregulation of these microRNAs decreased Jmjd6 levels at the onset of Ras expression. However, at later stages of melanoma progression, Jmjd6 levels were found elevated, suggesting that the microRNAs targeting Jmjd6 may be part of an anti-cancer response activated by Ras. Similar levels of the three microRNAs and JMD6 were also found in mouse models, human cancer cell lines and melanoma tissue obtained from several patients (Anelli et al., personal communication), thus confirming the potential of zebrafish melanoma as a model to identify novel molecular mechanisms in human diseases.

Intriguingly, a recent paper [29] reported that JMJD6 and bromodomain-containing protein 4 (BRD4), acting as functional partners, regulate Pol II promoter proximal pausing in a large subset of genes based on their actions on distal enhancers, termed anti-pause enhancer. In particular, JMJD6 and BRD4 retain P-TEFb complex (a heterodimer consisting of the cyclin-dependent kinase Cdk9 and a cyclin component), leading to subsequent Pol II phosphorylation and efficient transcriptional elongation. This finding is of particular interest as transcriptional elongation has been found to be involved in neural crest specification and melanocytes maturation [30], thus connecting results obtained from different studies in zebrafish melanocytes and melanoma.

In order to identify modifiers of RAS-induced melanomagenesis, we developed a screening strategy based on somatic expression of the oncogene and candidate modifiers using the *kita*:gal4 line and a UAS vector to express candidate modifier genes (Zettler et al., in preparation), including epigenetic modifiers. The screen helped to focus on JMJD6 as a microRNA target and RAS cooperating factor. Figure 1 shows a comparison of the two screening methods for melanoma modifiers described in this section.

Rac1 in Melanoma

The Rho-like GTPase Rac1 stimulates cellular processes such as proliferation, survival, motility and invasion [31], which are deregulated in cancer. Its function may be required for Ras-induced transformation, and recent NGS data identified RAC1-activating mutations as drivers in approximately 5 % of B-RAF and N-RAS negative cutaneous melanomas [18, 32].

Dalton and colleagues [33] contributed to understanding the initial steps of Ras-induced transformation by dissecting the role of *Rac1* in melanoma initiation and progression using a zebrafish melanoma model, where oncogenic H-RAS is expressed in melanocytes under the *mitfa* promoter. Results obtained in this study demonstrated that the co-expression of a constitutively active form of RAC1 (V12RAC1) and oncogenic RAS accelerates nodular tumor formation. Moreover, they found that the Rac activator Tiam1 (T cell lymphoma invasion and metastasis 1) is overexpressed in both zebrafish and human melanoma, suggesting that Tiam1 may activate Rac1, specifically in nodular tumors.

Mitf Levels in Melanoma

One of the important genes in melanocyte development and melanoma is the conserved “master melanocyte transcription regulator” microphthalmia-associated transcription factor (MITF) [34•]. This pivotal transcription factor is expressed in many melanomas, although its levels vary between specimens.

A temperature-sensitive *mitfa* zebrafish mutant was used to modulate endogenous MITF activity in melanoma in vivo [35]. This mutant revealed two important aspects regarding the role of Mitf in melanoma: first that low levels of endogenous Mitf activity cooperate with BRAF^{V600E} to promote melanoma and, second, that the complete absence of Mitf activity in BRAF^{V600E} melanoma leads to dramatic tumor regression, thus suggesting that Mitf acts as a rheostat in melanocyte transformation. These observations are in accordance with in vitro data in human cells showing that MITF works as a rheostat, with low levels of functional MITF present in G1-arrested melanocyte stem cells and elevated levels present in G1-arrested differentiated melanocytes or associated with proliferation of stem cells [36]. However, studies of MITF level manipulation in vivo were missing, and this clever zebrafish model provided important insights into MITF biology in melanoma. The study carried out in zebrafish could be relevant to find therapeutic solutions for human melanoma, as they clearly show that targeting MITF activity is a potent anti-tumor mechanism. On the other side, they also reveal that partial

Fig. 1 Screening strategies to identify genetic modifiers of melanoma development in adult (**A**) or larval (**B**) zebrafish models. **A** Candidate modifiers are placed under the *mitfa* promoter in the MiniCoopR vector (**a**) and injected (**b**) for expression in *tg(mitfa:BRAF^{V600E};p53^{-/-};mitfa^{-/-})* 1 cell embryos. **c** Injected larvae are screened for mosaic expression of *mitfa* and the candidate gene (black spots). **d** Selected larvae raised to adults are scored for melanoma development. **e** Melanoma-free survival curves are built on several observations at different time points. Modified from [17, 18]. **B** Candidate modifiers are placed under the *UAS* regulatory sequences for expression in the *kita:gal4* transgenic line to identify modifiers of

ras-induced melanomagenesis. **a** Different fluorescent markers identify Ras and candidate genes. The strategy is the same for controls (**b**), injected only with UAS:eGFP- HRAS^{G12V} (Ras), or for candidates (UAS:gene X or UAS:gene Y), co-injected with UAS:eGFP- HRAS^{G12V} (**c**, **d**). After injections, 1 dpf (day post-fertilization) embryos are selected for the expression of Ras (green fluorescent melanocytes) and candidate modifier gene (blue fluorescent nuclei, plus green heart). At 5 dpf, selected larvae are evaluated for the number of melanocyte spots. **e** Counts are plotted for the number of larvae with 1–5 spots, 6–10 or >10. Injections with Ras alone give on average 4–5 spots per larva (Zettler et al., in preparation)

or ineffective targeting of MITF is oncogenic, so drugs that are not able to completely abrogate MITF activity could be dangerous as they can promote the disease.

Telomerase

Increased telomerase activity has been detected in a large number of cancers [37]. As recently shown by Huang et al. [19], mutations of the TERT promoter carrying the classical UV-light signature (C > T) were found to be present in the majority (up to 70 %) of cutaneous melanomas. These mutations generate *de novo* binding sites for ETS transcription factors, leading to increased expression of the

catalytic subunit of telomerase and telomere elongation/maintenance in proliferating melanoma cells. In zebrafish, the BRAF^{V600E}/p53-transgenic melanoma model in the telomerase mutant background (*hu3430* [38, 39]) has a reduced melanoma burden (Ferreira MG et al., personal communication), thus showing the importance of telomere lengthening mechanisms in proliferative diseases in human and zebrafish.

Mutational Analysis of Zebrafish Melanoma Models

The exome of 53 B-RAF^{V600E} and N-RAS^{Q61K} zebrafish melanomas has recently been sequenced at the Sanger

Center and the results reported by Yen et al. [40•]. Surprisingly, the mutational landscape in these models, which are not exposed to UV light, revealed a UV light signature in an overall low mutation burden. Among the most interesting variations, amplification of a region encoding for a subunit of protein kinase A (*prkaca*) was found in 13 genomes, providing evidence of an involvement of PKA signaling in cooperation with MAPK and p53 loss in these melanoma models.

Chemical Screens and Translational Impact

Due to its small size, large progeny clutch and embryonic transparency, zebrafish serves as a superb in vivo animal model for chemical compound screens and characterization (reviewed in [41]). Ever since the first zebrafish-based chemical screen was conducted in the year 2000 [42], many functional chemicals have been discovered using this strategy. Due to the fact that melanocytes in zebrafish are easy to observe under a microscope, many screens have been performed by incubating the embryos in the presence of drugs at sphere stage (4–6 h post fertilization, hpf), well before the neural crest develops, and observing the effects on melanocyte number, size and migration compared to untreated control at the end of the incubation (usually 48 hpf). By screening 1,400 compounds from three different libraries at 10 μ M final concentration, 50 small molecules were identified that affect a range of pigment cell processes including specification, migration, proliferation and differentiation [43]. Roscovitine, fiduxosin and cyclooxygenase (COX) inhibitors were of particular interest from this screen, not least because they are currently used in humans to treat a range of conditions. In particular, roscovitine, a cyclin-dependent kinase inhibitor, being developed as an anti-cancer, anti-viral and anti-inflammatory drug, specifically interfered with the numbers of melanocytes in the developing embryos. Fiduxosin, which is used in clinics as a muscle relaxant, affected an iridophore differentiation transcriptional program, and COX inhibitors were able to cause spindly melanocytes at lower concentration or spot-like melanocytes at higher concentration [43].

Another chemical screen of 6,000 compounds identified two compounds inhibiting pigment formation in zebrafish, 12G9 and 36E9 [44]. TUNEL assay indicated that these two compounds induced apoptosis of melanocytes, a mechanism of action particularly attractive for treating melanoma. Moreover, 12G9 was able to inhibit the viability of a murine melanoma cell line but had no effect on a human pancreatic carcinoma cell line, thus showing its specificity for melanocytes.

Finally, with the aim to identify small-molecule suppressors of neural crest progenitors, White and colleagues

[30] screened 2,000 small molecules using as a readout crestin expression (a neural crest-specific marker [45]) by in situ hybridization. The rationale for study pathways involved in neural crest specification came from the notion that melanocytes are originally derived from the embryonic neural crest. Moreover, in adult zebrafish melanoma, almost all tumor cells are positive for crestin [30], and the gene expression signature of BRAF^{V600E}/p53-adult melanoma overlaps with that of crestin transgenic cells in the embryo. Neural crest progenitors were also found in a human melanoma tissue array [30].

One class of compounds, inhibiting dihydroorotate dehydrogenase and including leflunomide, was able to completely abrogate neural crest development in zebrafish embryos. Leflunomide inhibits the transcriptional elongation of genes that are required for neural crest development and melanoma. Intriguingly, leflunomide, in combination with a specific inhibitor of BRAF^{V600E}, led to a marked decrease in melanoma growth in both in vitro and mouse xenograft models. This study could have important implications for understanding pathways involved in the initial transformation events in melanoma and for therapy.

Other Developments of Fish Melanoma Models

The availability of several transgenic lines where specific cell lineages such as endothelial cells or leukocytes are marked with fluorescent proteins and the existence of zebrafish compound mutant mostly transparent also as adults (*casper* [46]) makes zebrafish a powerful vertebrate model to visualize and manipulate in vivo inflammation, angiogenesis and metastasis in the context of melanoma.

Melanoma and Immune Cells

It is known that the immune system is essential for various steps of cancer initiation and progression, as it can both identify and target transformed cells. However, it can also be an active participant that can aid the expansion and metastatic spread of a tumor. However, to detect the earliest immune system-tumor interactions in murine models is challenging, as transformed cells cannot be predictably traced in the organism. In the zebrafish, cells of the innate immune system emerge as early as 15 hpf and can respond to infections and wounds from around 22 hpf [47, 48]. However, cells of the mature adaptive immune system emerge later, at around 1 week of larval life [49, 50], giving us the advantage of distinguishing between the contribution of the innate immune cells and the adaptive immune system in various processes involving the immune system in larval zebrafish. Moreover, zebrafish transgenic lines that have fluorescently labeled leukocyte subtypes in

different colors are available [51–53], allowing easy in vivo observations under a fluorescent microscope. Zebrafish melanoma models were used by Feng [54] to delineate possible early interactions between oncogene-transformed melanoblasts and goblet cells and the immune system in the skin of zebrafish larvae. Results of this study indicate that transformed cells can activate and recruit host leukocytes from very early developmental stages. In addition, the initial recruitment to transformed cells is, at least partially, triggered by local H_2O_2 synthesis. These data reveal a previously unknown homology between the transformed cell-induced host innate immune response and a wound inflammatory response.

Two years later, the same author [55•], using both genetic and pharmacological approaches, found that the COX-2/PGE₂ pathway is involved in the process of expansion of transformed cells. In particular, at early stages of cancer initiation, leukocytes generate PGE₂, and this mediator promotes the growth of transformed cells via the specific receptor EP1, expressed in transformed cells. Furthermore, using a zebrafish transgenic line in which macrophages are labeled by low levels of a NFκB reporter, they showed that reduced PGE₂ production leads to altered neutrophil migration and increased cell death of transformed cells, followed by their rapid engulfment by macrophages. These in vivo observations in zebrafish embryos at the early stage of cancer initiation provide mechanistic understanding for the reported effect of non-steroidal anti-inflammatory drugs in reducing cancer incidence [55•, 56].

Zebrafish as a Tool to Study Melanoma Metastasis

Melanomas are highly metastatic skin tumors where the presence of lymph nodes and visceral metastasis is directly related to poor outcome, with a mean survival time of 7.5 months from diagnosis [57]. Metastases result from a combination of several mechanisms including epithelial-mesenchymal transition, loss of cell-to-cell adhesion, loss of cell–matrix adhesion, matrix degradation, chemo-attraction/repulsion and migration [58]. Although several signaling pathways, including MAPK, PI3K and Wnt/β-catenin [59], have been implicated in melanoma metastatic spread, many questions remain still open, and studies uncovering molecular pathways involved in melanoma metastasis in vivo are needed. Zebrafish has proved to be a great model due to its transparency at larval stages. However, as in murine and human systems, the in vivo spatial resolution of the adult zebrafish is limited because of the normal opacification of the skin and subdermal structures. To overcome this problem and use the zebrafish to study metastasis in adult animals, a transparent zebrafish compound mutant, called *casper*, lacking melanocytes and iridophores, was generated [46]. Tumors carrying either the mutated human B-RAF or

N-RAS-GFP fusion derived from adult fish were disaggregated into a single-cell suspension and transplanted either into the ventral peritoneum or via intraventricular injection into the irradiated *casper* recipient. Using this approach, White and colleagues found that groups of tumor cells expressing GFP are present in sites distant from the injections [46]. Importantly, in these fish, melanomas and their derived metastases can be imaged and quantified over long periods using standard stereomicroscopy without the need to sacrifice the animal. This model opens the doors to future studies aiming at understanding the factors that determine the capacity of melanoma cells to undergo early dissemination as well as which signaling pathways are involved in specific organ colonizations, which will be facilitated by the development of isogenic fish lines [60].

Many studies indicate chemokines as important signals in tumor progression and metastasis. In human melanoma, high levels of the chemokine receptors *cxcr4* and *cxcr7* have been reported [61, 62]. Moreover, several in vitro and mouse models of chemokine signaling indicated a small set of chemokine receptors guiding organ-specific melanoma metastasis, including *Sdf1/Cxcr4* [63]. The medaka melanoma model, where the oncogene *Xmrk*, a homolog of the human epidermal growth factor receptor, is expressed in melanocytes under the *mitf* promoter, was used to investigate the influence of *Sdf1* chemokine signaling in melanoma progression [64]. Results obtained using this model showed that *Sdf1* signaling via *Cxcr7* is able to constrain melanoma growth in vivo and that these signals influence tumor outcome.

Conclusions

The complexity of melanoma is nicely reflected in the fish models, which are now starting to provide novel insights into the pathobiology of the disease. Newly discovered determinants of melanoma development are scrutinized in fish genetic models, where the cells of origin, the molecular drivers and the biology of transformed cells in a whole organism are carefully controlled and monitored through the evolution of the disease. These models are continuously updated to reflect data obtained from molecular and cellular snapshots of human cases and in the future will also include the response to drug treatments, which is one of the most urgent demands on the experimental cancer research community.

Compliance with Ethics Guidelines

Conflict of Interest Viviana Anelli, Nicole Zettler and Marina Mione declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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Recently published papers of particular importance have been highlighted as:

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