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NEW INSIGHT IN MELANOMA STUDIES FROM THE ZEBRAFISH ANIMAL MODEL

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Abstract – *Zebrafish is a very versatile model to analyze the function of specific cancer-related genes via different approaches, such as modulation of gene expression, gene knockout, generation of transgenic lines and tumor cells transplantations. Moreover, tumors show a relevant degree of histological similarity compared to those present in humans. This explains how, in the past years, the zebrafish has become one of the most powerful preclinical models for mimicking different human diseases, in particular, tumors onset and spreading. This review highlights the importance of the zebrafish model for cancer research, in particular providing insights into the state of art of this teleost as a model for the study of melanoma biology.*

Despite the differences between zebrafish and human epidermis, the melanomas affecting both species are very similar. In addition, humans and zebrafish share the developmental program that specifies for the formation of melanocytes. It was shown that, in zebrafish, the genetic regulation of melanocytes specification relies upon the nacre/mitfa gene, direct target of the Wnt signaling; moreover, the serine/threonine kinase BRAF is involved in nevi formation such that human BRAF mutations were found to be associated with melanoma development both in humans and fish. A more recent work has shown how the zebrafish can be exploited to follow melanoma development in vivo from its onset as a single cell, demonstrating that tumor occurrence is accompanied by the reemergence of a neural crest progenitor state in the cells that will originate the malignancies.

Further exploitation of the zebrafish melanoma model will be instrumental for a better understanding of this disease and for developing focused therapeutic strategies.

KEYWORDS: *Animal models, Zebrafish, Carcinogenesis, Melanoma, Progenitor state reemergence.*

INTRODUCTION

Nowadays, animal models play an essential role in mimicking and elucidating the mechanisms of tumors onset and spreading, as well as in paving the way for establishing new therapeutic approaches, which are always becoming more cancer-stage-specific and cancer-type-specific.

The teleost (bony) fish zebrafish (*Danio rerio*) is, to date, one of the most widespread aquatic pets and its use as a model organism was pioneered by Dr. George Streisinger at the University of Oregon[1]. In the past decades it has become a very common and valuable model in biological research and, these days, it is considered one of the most reliable animal species for the study and understanding of the most fundamental vertebrate developmental dynamics[2-4]. Procedures for its staging and raising have been standardized and are routinely used today by researchers all over the world[5]. Among the main advantages of this vertebrate system, it is worth mentioning its short generation time, its high fecundity (up to 200 eggs per mating), the external fertilization, the

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transparency of its small size embryos developing outside the mother's body and the possibility of manipulating and growing them very easily under standard conditions[1,5,6]. The sequencing of its reference genome has been completed and, more importantly, approximately 70% of the human genes have at least one zebrafish orthologue[7]. All these features make the zebrafish a very practical and inexpensive model.

ZEBRAFISH AS A MODEL SYSTEM FOR CANCER RESEARCH

One of the most used and efficient approaches is to exploit the animal model to experimentally induce the development of a particular cancer type, which requires that the model closely resembles human cancer. This is very useful to study the onset of cancer as well as its evolution in time and, to note, this strategy also allows us to conduct large drug discovery screens.

The zebrafish, initially exploited above all for elucidating the dynamics controlling early embryo development[8-11], recently turned out to be powerfully useful for understanding the molecular mechanisms involved in several human diseases[12-18], *in primis* tumor occurrence and progression[19-21]. Indeed, despite its higher phylogenetic distance compared to other mammalian models, many human cancer cell types have been modeled in the zebrafish, due to the fact that the molecular pathways that regulate cell divisions and proliferation are evolutionarily conserved among mammals and non-mammalian vertebrates[22-25]. To note, the developmental and genetic programs often misregulated in cancer are largely shared by zebrafish and mammals[26].

Although fish do not possess all organs present in mammals, zebrafish organs are functionally and morphologically similar to the human ones and, moreover, tumors originating in fish tissues show a very high and significant histological similarity compared to their human counterparts[22,27]. Similarly, the tumor-inducing angiogenic mechanism seems to be also conserved in zebrafish[28,29]. Importantly, this model system was found to be especially prone to spontaneously develop a large number and variety of tumors after exposure to carcinogens[30,31].

Different techniques have been successfully established and are nowadays available to deepen the analysis of the function of specific cancer-related genes or to induce tumors and thus have the possibility to follow their progression using the zebrafish model.

The forward genetic approach via treatment with chemicals, irradiation or insertional mutagenesis has been classically used for large-scale mutagenesis screens, with the aim to identify vertebrate genes responsible for the development of the embryo[32-34]. Interestingly, many of the pathways involved in embryo development and targeted by the aforementioned experimental approaches, if dysregulated in the adult animal, are directly involved in cancer induction and development. Thus, zebrafish became also one of the most suitable vertebrate models for modulating the expression of cancer-specific genes and evaluating their effect, for example during the first stages of embryonic development[22]. The most efficient ways to modulate gene expression in zebrafish are morpholino-mediated knockdown and overexpression through mRNA injection[21,35-37]. In a large number of studies, the transposon-mediated approach turned out to be very successful for the establishment of consistent transgenic zebrafish models of tumor progression, also thanks to the use of tissue-specific promoters to switch on or off the expression of specific genes selectively in the tissues of interest[38].

More recently, the genome editing technique based on the CRISPR-Cas9 system has made reverse genetics approaches in zebrafish more efficient and affordable, with the possibility of obtaining a large number of mutants with radically reduced efforts in terms of time and laboratory resources compared to the previous mutagenesis methods, thus permitting to evaluate the effects of specific gene mutations on cancer development[39-44]. In parallel with the spreading of the CRISPR-Cas9 technique in all the most important zebrafish laboratories around the world, it became clear that a lot of mutants were not phenocopying at all the results previously obtained with morpholino, thus raising a lot of concerns about the specificity and reliability of oligonucleotide-mediated knockdown analysis[45,46]; however, it was then shown that a process of genetic compensation occurring in mutants but not in morphants could very well explain this discrepancy[47] and morpholino-mediated knockdown approach, if properly validated and confirmed with all the necessary controls[48,49], is still considered a valid experimental plan for modulating gene function during zebrafish embryonic development.

The zebrafish model also gives the powerful possibility to perform specific tumor cells transplantations, behaving as a recipient where tumor cells deriving from a donor can be grown[20]. This technology, called xenograft, allows us to study human cancer cells behavior *in vivo*, namely to determine the ability of these cells to grow and metastasize by inducing angiogenesis[28,50].

All these features establish the zebrafish as a unique *in vivo* system for modeling human can-

cers. Initially, it turned out to be a very useful and predictive model for the study of non-solid tumors; its importance in that direction has already been demonstrated extensively, for example for what concerns leukemia models[51-55]. Later, with the establishment of the homozygous mutant fish for the oncosuppressor factor *p53*[56], it also became extremely suitable for the analysis of solid tumors onset and spreading, due to the higher frequency of tumor occurrence in this line; among these types of tumors, one of the most studied and best characterized is certainly melanoma.

Here, we review recent literature about the relevance of the zebrafish for the modeling of solid tumor onset and spreading, taking melanoma as an example, highlighting how this system can provide very useful insights for the study of endogenous tumors.

THE ORIGIN OF NEVI AND MELANOMAS: THE ZEBRAFISH MODEL

A deep understanding of the mechanisms underlying the development of the melanocyte lineage since its early specification is fundamental as it could very likely provide essential insights about genetic pathways that might be altered during melanoma occurrence; indeed, a lot of genes involved in melanocyte specification and development are known to play pathogenic roles during cancer onset and, in general, different advanced malignancies were shown to restart the expression of embryonic genes[57,58].

To this aim, in the past years the zebrafish turned out to be a powerful model for the study of melanoma biology[59]; in fact, zebrafish melanomas are strikingly similar to their human counterparts, for what concerns both genetic features and phenotypic characteristics. Moreover, in this model system, melanomas are very easily detectable, thanks to the fact that this animal does not present consistent adipose tissues that could mask the tumor mass, as happens for example in rodent models. Zebrafish melanocytes are externally visible, and single cells can be easily visualized in a living animal[60].

In zebrafish the melanocytes, like all subtypes of pigment cells, derive from the highly motile population of the neural crest (NC) cells, a temporary embryonic structure induced during gastrulation by Wnts-, BMPs-, FGFs- and Notch-dependent signaling events, originating from the ectoderm cell layer and giving rise to different cell lineages[61-64]. Most NC cell fates are already specified very early during development, prior to the starting of migration[65]. The NC-derived melanocyte differentiation program was demonstrated to be conserved among vertebrates[66]. Before migration, NC cells undergo a so-called epithelial-mesenchymal transition (EMT) process, during which they lose adhesion to the neighboring cells and become motile[60,67]. In zebrafish, melanocyte precursors start to express melanin around 24 hpf (hours post-fertilization), and the typical embryonic pattern of pigmentation is mostly completed by 48 hpf[66].

It was shown that Wnt signals are necessary and sufficient to direct the specification of neural crest to a pigment cell fate, this occurring before the migration process begins[68]. During zebrafish development, the key target gene activated by Wnt signals was identified in the *mitfa/nacre* gene, a transcription factor homologous to *MITF* expressed in differentiating neural crest-derived melanophores as well as in the retinal pigmented epithelium[69-71]. Importantly, functional inactivation of *mitfa/nacre* results in a failure of neural-crest derived melanocytes to differentiate[69,70]. The discovery that the promoter region of the zebrafish *mitfa/nacre* gene contains three Tcf/Lef (family of high-mobility-group transcription factors co-activated by the Wnt-dependent β-catenin accumulation in the nucleus) binding sites necessary for interaction with zebrafish Lef1 *in vitro* as well as for proper expression in zebrafish embryos identified *mitfa/nacre* as direct target of the Wnt signaling[70].

Among the factors that have been shown to be directly linked to the formation of nevi (groups of melanocytes clustered together) and to their malignant transformation to melanoma, the serine/threonine kinase BRAF appears to be one of the most relevant: *BRAF* or *RAS* mutations are a feature of approximately 80% of the cases[72-76]. Patton et al[73] were able to establish a zebrafish model for melanoma development by analyzing BRAF function and *in vivo* monitoring nevi formation. The authors used a stable transgenic approach to drive the expression of the human *BRAF* gene carrying the mutation most commonly found in human nevi and melanoma ($BRAF^{V600E}$) specifically in the epidermis of the fish, placing it under the control of the melanocytes-specific promoter *mitfa/nacre*[70]. They were in this way able to demonstrate that the expression of this mutant form of *BRAF* led to the occurrence of ectopic melanocytes and nevi in the fish, starting from 8 weeks post-fertilization[73]. Moreover, the same transgenic approach performed using a *p53*-deficient zebrafish background resulted in a significant increase of melanocyte lesions that were highly prone to degenerate into invasive melanomas, thus demonstrating that the BRAF factor, in a *p53*-depleted

environment, is sufficient for nevi formation and it represents one of the primary players during melanoma occurrence.

MELANOMA INITIATION REACTIVATES NC GENETIC PROGRAM

A recent work carried out by Kaufman et al[77] using the zebrafish model was able to further elucidate the key molecular steps occurring during melanoma initiation, at the same time giving the possibility, in the near future, to consider new interesting perspectives in the development of preventive therapeutic approaches against aggressive malignancies such as melanoma[72].

The authors were able to exploit the zebrafish model in order to monitor *in vivo* the events underlying melanoma initiation, taking advantage of the particular expression pattern of the *crestin* gene, already known to be a marker of the zebrafish neural crest and to be specifically re-switched on in adult melanoma tumors[77-79]. First of all, they proved that melanoma cells re-gain expression of the neural crest marker *crestin*, by successfully establishing a transgenic line expressing the zebrafish *crestin* gene upstream of an EGFP reporter. This experiment was conducted using as genetic background the triple transgenic line *p53*-deficient/*nacre:BRAF*^{V600E}/*crestin:EGFP*, prone to developing invasive melanomas[73,77]. Interestingly, the authors observed that some patches of cells started to express *crestin:EGFP* before the onset of melanoma lesions; these EGFP^{+} cells were demonstrated to be tumorigenic, as they were able to survive and expand locally after transplant, suggesting that this population reactivating *crestin* expression, and thus the neural crest progenitor (NCP) state, could represent a specific subset of cells that, within a tissue of cancer-prone cells, are pre-specified to undergo the transition to a malignant state[77].

Next, through a specific binding site-mutagenesis approach, the researchers identified *sox10*, a well-known neural crest developmental regulator, as the main transcriptional activator of *crestin* expression. Moreover, gene analysis expression by microarrays showed that individual zebrafish scales carrying early *crestin*:EGFP patches, thus prone to develop melanoma, were significantly enriched in neural crest regulator and melanoma specific transcripts, like *sox10*. The authors also performed a *sox10* functional analysis, showing that its overexpression, specifically induced in melanocytes, resulted in an accelerated melanoma formation, whereas *sox10* knockout by CRISPR/Cas9 technology was able to significantly inhibit cancer occurrence.

Interestingly, the authors of this work showed that the expression of NCP genes in neural crest cells and both zebrafish and human melanomas was correlated to the activation of specific super-enhancers, in the context of a complex epigenetic control, leading to the reemergence of a NCP identity in these particular cellular populations.

Altogether, the results presented in this study demonstrate that melanoma precursor cells, both

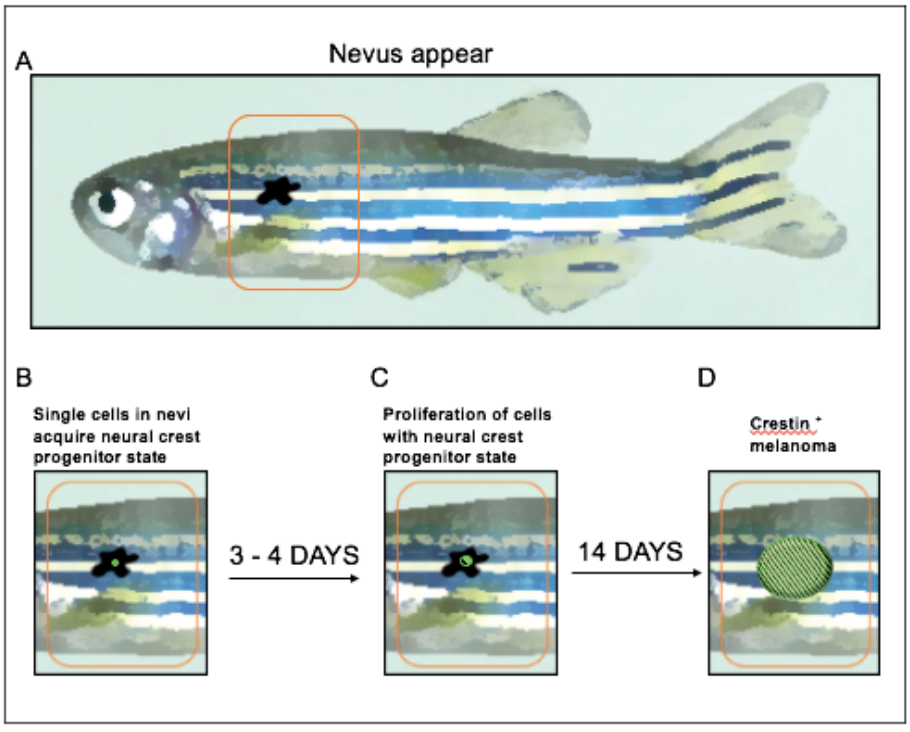

Fig. 1. Main steps of melanoma development in zebrafish. *A*, Arising nevus in a *p53*-deficient/*nacre:BRAF*^{V600E}/*crestin:EGFP* fish line. *B*, One cell within the nevus restarts to express typical neural crest progenitor genes, such as *crestin*. *C*, Proliferation and transformation into a tumor of the single *crestin*:EGFP^{+} cell present in B. *D*, Complete development of melanoma: all cells are enriched in neural crest markers expression *(crestin)*.

in human and zebrafish, restart an embryonic neural crest developmental program and that this reemergence of NCP-specific features represents a key step occurring during cancer initiation. Hence, these data show how only sporadic cells within a larger nevus present a gene expression profile that allows the transition to the malignant state. The authors called this subpopulation "cancerized field". According to them, the acquisition of a tumor fate is very likely due to a combination of gene dysfunction occurring within the single cells together with aberrant signals originating from the niche environment[77] (Figure 1). Remains to be investigated how much backward in the NC developmental program investigators will be able to go to find the original genetic step leading to melanoma induction. For instance, would it be Wnt, BMP or Notch signaling or a combination of those to affect the "cancerized field" such to induce the transition to a malignant state?

Kaufman et al[77] demonstrated how the zebrafish could be very useful to establish a live-image model of cancer initiation from its origin as a single cell, showing the importance to combine in the same model the analysis of the cellular processes occurring during vertebrate embryonic patterning and the events underlying cancer occurrence and growth.

CONCLUSIONS

Among solid cancers, melanoma is surely one of the most devastating and lethal. Great steps forward have been made in the last years towards the discovery of the molecular pathways involved in the onset of this and other types of cancer diseases, as well as in metastasis occurrence. Animal models, like the zebrafish, are playing a fundamental role for the modeling of human malignancies and several important results have been obtained in the past years. Thanks to its unique features, zebrafish is a candidate for becoming the ideal preclinical model for melanoma cancer studies; further research in the field of drug screening is now required to be able to develop specific therapeutic approaches[80,81] for the treatment of this disease and to improve patient prognosis. We are confident that the zebrafish community of researchers will give a fundamental contribution to this aim.

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Conflict of interests

The Authors declare that they have no conflict of interests.

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