REVIEW

Fishing for neuroendocrine tumors

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Abstract

Neuroendocrine tumors (NETs) are a class of rare and heterogeneous neoplasms that originate from the neuroendocrine system. In several cases, these neoplasms can release bioactive hormones leading to characteristic clinical syndromes and hormonal dysregulations with detrimental impact on the quality of life and survival of these patients. Only few animal models are currently available to investigate pathogenesis, progression and functional syndromes in NETs and to identify new therapeutic strategies. The tropical teleost zebrafish (*Danio rerio*) is a popular vertebrate model system that offers unique advantages for the study of several biological processes, ranging from embryonic development to human diseases such as cancer. In this review, we summarize recent advances on zebrafish models for NET preclinical research that take advantage of modern genetic and transplantable technologies. In the future, these tools may have a role in the treatment decision-making and tertiary prevention of NETs.

Introduction

Neuroendocrine tumors (NETs) represent a broad class of neoplasms originating from neuroendocrine cells. NETs can cause a wide array of symptoms depending on the type of tumor, its location and the production of several factors. In functioning tumors, the release of several bioactive hormones can lead to characteristic clinical syndromes and hormonal dysregulations, with detrimental impact on the quality of life and survival of these patients. Non-functioning NETs are the majority of tumors. They do not release bioactive hormones and are often clinically silent for a long time. As a result, non-functioning NETs are diagnosed in the later stages after the occurrence of symptoms related to the mass effect of the tumor or metastases. (Rindi & Wiedenmann 2011, De Angelis *et al.* 2018). Although surgery remains the cornerstone of treatment for localized tumors, most patients with NETs are diagnosed when they already have

metastases, because these neoplasms are often indolent. In advanced disease, the efficacy of current medical strategies is limited by the high biological heterogeneity of these neoplasms in terms of clinical aggressiveness and response to the therapy (Uri & Grozinsky-Glasberg 2018, Alexandraki *et al.* 2019).

In this context, new animal models that faithfully recapitulate clinical features and related complexity of NETs are needed for the development of innovative therapeutic strategies and to clarify the mechanisms involved in tumor progression. Although rodents represent the main animal model in cancer research, the use of this model in the field of NETs is very limited. In the last decade, the use of zebrafish (*Danio rerio*) in biomedical research is growing exponentially, with relevant applications in studying human diseases (Lohr & Hammerschmidt 2011), such as cancer modeling (Astell & Sieger 2019,

Hason & Bartunek 2019, Osmani & Goetz 2019). In 2014, we have published an article providing a comprehensive overview of zebrafish in NET research, describing genetic models and our preliminary results of NET xenotransplantation in zebrafish embryos. In the present review, we provide an update on these models, underlying how the availability of multiple experimental strategies makes zebrafish extremely versatile in the NET research.

Zebrafish model in cancer research

The teleost zebrafish has emerged as a relevant *in vivo* model for research in genetic and embryology. The appeal for these animals lies in the high fecundity, the outer fertilization, the rapidity of embryonic and larval development and the optical transparency of zebrafish embryos. Moreover, compared to other vertebrate models, adult zebrafish are very easy to maintain under laboratory conditions because of their size and the possibility to keep them in relatively high density. More recently, the focus of zebrafish research has progressively shifted toward topics that are also relevant for human diseases, including tumors (Santoriello & Zon 2012, Shive 2013, Vitale *et al.* 2014, Gaudenzi *et al.* 2017, Peverelli *et al.* 2017, Wurth *et al.* 2017, Cirello *et al.* 2018).

Although zebrafish can develop tumors in various organs with high degree of histological and molecular conservation compared with human malignances (Stern & Zon 2003), their spontaneous incidence is very low. However, alternative experimental approaches have been recently developed in zebrafish to study both genetic basis of cancer as well as tumor progression.

To generate genetic models of cancer, several forward and reverse strategies have been used in zebrafish. Through large scale forward genetic screening it is possible to identify cancer susceptibility genes, responsible for a specific and well-characterized phenotype. After the induction of random modifications throughout the genome, by carcinogens, irradiation or viral/transposon-based vectors, progeny can be easily screened for cancer phenotypes, taking advantage of embryonic and larval transparency. Causative mutations can be identified through genetic mapping and sequencing analysis. The rapid development of zebrafish genomic resources has promoted the identification of complementary reverse genetic approaches to investigate genes and pathways of interest. Compared to forward strategies, reverse genetic approaches are based on gene manipulation and transgene introduction into zebrafish genome,

such as human genes with cancer-associated mutations, with the aim of generating tumor-related phenotypes. A reverse genetic approach, commonly used to study cancer-related genes in zebrafish embryos and larvae, is based on their transient knockdown or overexpression (Finckbeiner *et al.* 2011, Kim *et al.* 2017, Grosse *et al.* 2019). The transient gene knockdown strategy relies on the injection of specific morpholinos (MOs), synthetic antisense oligonucleotides in which the replacement of RNA ribose rings by morpholine rings prevents nuclease digestion. MOs, typically injected into embryos at the 1-cell stage, exert their knocking down action by binding complementary target mRNAs, thus preventing their translation or splicing. The transient overexpression during early zebrafish development (up to 3 days) is achieved by introducing the mRNA encoding the protein of interest into the embryos during the first 2 h of development. Given that MOs and exogenous RNAs are efficacious only few days after the injection, these techniques are of short duration and not suitable for functional studies beyond the larval period (Nasevicius & Ekker 2000, Bill *et al.* 2009). Nevertheless, MO technology is adequate to study several developmental and cellular processes and molecular pathways that are also related to cancer biology (Amatruda *et al.* 2002, Hason & Bartunek 2019). For instance, it has been reported that aggressive tumor cells show aberrant activation of embryonic signaling, such as *nodal* and *notch* pathways, leading to a multipotent phenotype similar to embryonic stem cells (Strizzi *et al.* 2009). Also, Wnt signaling has been tightly associated with both development and cancer (Zhan *et al.* 2017). In this frame, the possibility to easily modulate the expression of novel Wnt signaling regulator during early zebrafish development by means of MO technology (Kim *et al.* 2017, Grosse *et al.* 2019) represents a unique opportunity to investigate aberrant molecular events involved in carcinogenesis.

Cancer modeling in zebrafish can also rely on numerous mutant and transgenic lines that allow study of cancer-related phenotypes in a broader temporary window (Shive 2013). Several strategies are currently available to create mutant lines in zebrafish. They are based on the possibility to generate double-strand breaks at specific sites in the zebrafish genome that can be imprecisely repaired by non-homologous end joining (NHEJ), a DNA repair pathway that frequently causes small insertions or deletions at the break site. One of these strategies is based on Zinc finger endonucleases, in which a DNA-binding zinc finger protein is fused to a nonspecific cleavage domain of the FokI endonuclease. Upon binding to a specific DNA

sequence by the zinc-finger motifs, FokI endonuclease can induce double-strand breaks that can be imprecisely repaired by NHEJ (Santoriello & Zon 2012, Shive 2013). Another strategy for genome engineering is based on TALENs, chimeric nucleases generated by a transcription activator-like effector DNA-binding domain, constructed to bind any desired DNA sequence fused to a DNA cleavage domain (Santoriello & Zon 2012, Shive 2013). At the moment, the most used strategy for the genome editing is CRISPR–Cas9, an adaptive immune system used by bacteria and archaea against invading foreign nucleic acids derived from bacteriophages or exogenous plasmids. A chimeric single guide RNA is synthetized to interact with the complementary strand of the DNA target site, close to protospacer adjacent motif sequence, which is recognized and cleaved by Cas9 protein (Liu *et al.* 2017).

Another strategy to generate genetic models of cancer in zebrafish is based on transgenic animals in which tissue-specific promoters regulate the expression of murine or human oncogene, in both WT and mutated form (Santoriello & Zon 2012). In order to improve degree and precision of temporal and spatial expression of exogenous genes, several technologies have been adopted, such as Tol2 transposon and the mifepristone-inducible LexPR, GAL4-UAS and Cre-LoxP systems (Santoriello & Zon 2012). Moreover, it has been recently demonstrated that transgene electroporation can allow the spatio-temporal expression of specific oncogenes directly into adult somatic tissue (Callahan *et al.* 2018).

A limitation of both transient and stable genetic cancer models is related to the duplication that occurred in the stem lineage of teleost (Postlethwait *et al.* 2000). Considering that at least 20% of duplicated gene pairs may be retained from this event (Postlethwait *et al.* 2000), several human genes have more than one ortologue in zebrafish, leading to an extra work to investigate their specific functional roles and difficulties to reproduce the molecular conditions of human patients in zebrafish.

In addition to genetic basis of cancer, zebrafish offers the possibility to study several aspects of tumor progression (cell–stromal interactions, tumor-induced angiogenesis and metastasis formation) by performing xenotransplantation of human or mouse cancer cells in several sites of embryos, larvae, juvenile and adult fish. At present, embryo represents the most commonly used recipient for cancer xenograft assays in zebrafish. These studies can benefit from both intrinsic features of zebrafish model and the availability of transgenic lines that express fluorescent proteins in normal tissues, such as endothelium or immune system (Konantz *et al.* 2012,

Hason & Bartunek 2019). Although murine models remain the gold standard for xenotransplantation studies, tumor implant in zebrafish, and in particular in its embryos, can overcome some relevant drawbacks reported in mice (Zhao *et al.* 2015). For instance, maintenance cost of a zebrafish facility is lower than in mice and its management is simpler. The response to tumor implantation in zebrafish embryos, in terms of proangiogenic effects of implanted cells or their metastatic behavior, can be readily observed in real time and only after 24 h post injection (hpi), a time window narrower than that required in mice, ranging from few weeks to months. Immunosuppression is not needed because zebrafish embryos do not have a fully developed immune system, thus no graft rejection occurs at this stage of development. Besides, zebrafish offers the possibility to study the effects of small tumor implants (100–1000 cells/embryo), compared to larger implants (about 1 million cells) required in mice. In addition to the implantation of immortalized cell lines, zebrafish has been used as recipient for the injection of primary cultures, derived from post-surgical tumor samples (Vitale *et al.* 2014, Gaudenzi *et al.* 2017, Peverelli *et al.* 2017, Wurth *et al.* 2017, Cirello *et al.* 2018). These patient-derived xenografts (PDXs), largely employed in murine models, preserve the histological organization, the genetic and epigenetic mutational profile and the gene expression pattern, as in the patient counterpart. Due to these peculiarities, PDXs are currently considered a powerful platform for the development of precision medicine (Byrne *et al.* 2017). Recently, an elegant study has demonstrated that PDXs of human colorectal cancer in zebrafish embryos respond to the available therapeutic options as in patients (Fior *et al.* 2017). Thus, PDXs in zebrafish embryos (zPDXs) may open new frontiers in the personalization of anticancer treatment. Indeed, tumor xenografts in zebrafish embryos represent an advantageous platform to perform drug screening of new anticancer molecules. Because of the permeability of zebrafish embryos to small molecules, these drugs can be added directly to the embryo water, whereas larger or not water-soluble molecules can be injected into the blood circulation (Konantz *et al.* 2012, Fior *et al.* 2017, Hason & Bartunek 2019, Osmani & Goetz 2019).

Despite the described advantages, tumor xenografts in zebrafish embryos have few potential limitations that need to be considered. For instance, zebrafish embryos are maintained at 28°C and this may not represent an optimal temperature for mammalian cell growth and metabolism. Species-specific microenvironmental differences may affect the behavior of grafted mammalian

tumor cells. The lack of some mammalian organs in fishes (such as mammary gland, prostate and lung) precludes the possibility to perform orthotopic transplantations as in mice. Although embryonic organs and systems are completely defined, their differentiation is incomplete in embryos. This aspect together with the physiological differences between fish and mammals may influence drug metabolism in zebrafish, which may be different from that in mammals (Gaudenzi et al. 2019). Advantages and limitations in performing tumor xenografts in zebrafish embryos are summarized in Table 1.

Tumor xenografts can be performed also in juvenile and adult zebrafish. The availability of *casper* mutant strain, lacking all melanocytes and iridophores, offers the unique possibility to visualize tumor engraftment proliferation and metastasis formation in a large time window, from 5 days to 4 weeks, in adult fish (White et al. 2008). Moreover, the impact of the tumor graft on the mature vasculature of juvenile and adult zebrafish may better recapitulate tumor angiogenesis in cancer patients than embryos (Stoletov & Klemke 2008). Finally, in adult fish, pharmacological treatment and the drug delivery may be potentially similar to mouse models, in fact drug administration in embryo fish medium could not permit accurate drug dosing, optimized drug schedule and evaluation of pharmacodynamics over extended periods (Stoletov & Klemke 2008, Osmani & Goetz 2019).

The main limitation of tumor cell allografts and xenografts is that immune suppression is required to ensure the survival of implanted cells. To this purpose, chemical treatment with dexamethasone or sublethal doses of γ irradiation, (Langenau et al. 2004, Traver et al. 2004) can lead to a temporary ablation of the immune system in juvenile and adult zebrafish. However, these methods are not suitable for durable engraftment and consequently long-term tumor growth and dissemination analysis (Smith et al. 2010).

Alternatively, genetically immunocompromised fish, lacking the adaptive immunity, are currently available as tumor cell recipient. The first immunodeficient zebrafish line with the lack of mature T-cells and a reduction of B-cell number has been generated by Tang et al. (2014). New zebrafish immunodeficient models with affected T-cells, B-cells and natural killer (NK) cells have been recently developed (Moore et al. 2016, Yan et al. 2019). It has been demonstrated that a wide variety of tumor cell lines and patient-derived tumor cells grafted in these recipients have similar growth kinetics and histopathologic features to those grown in immunodeficient NOD scid gamma (NSG) mice (Yan et al. 2019). Therefore, these promising results support the use of adult zebrafish xenografts in the future of cancer research as a reliable preclinical model, comparable to the implantation in mice (Hason & Bartunek 2019, Yan et al. 2019).

To overcome transplant rejection in adult zebrafish without immune suppression, it is possible to perform allograft between clonal homozygous zebrafish. This procedure allows the transfer of tumor tissues from one donor fish to another syngeneic fish belonging to the same line (Mizgireuv & Revskoy 2006, Mizgirev et al. 2018). In this way it is possible to study tumor progression and tumor microenvironment over time in fish with fully functional immune system, but only between clonal fish.

The neuroendocrine system in zebrafish

Several lines of evidence indicate the conservation of neuroendocrine system in vertebrate, from fish to mammals, in terms of both morphological structures and their functions. As in mammals, the neuroendocrine regulation in zebrafish is based on the interconnection between structures of the CNS, such as hypothalamus and pituitary gland, and several peripheral organs including

Table 1 Advantages and limitations of tumor xenografts in zebrafish embryos.

Advantages	Limitations
High number of embryos can be implanted in the same experiment	The lack of several tissues and organs present in mammals limits the possibility of orthotopic implantation
Real-time and *in vivo* monitoring of proangiogenic potential and metastatic behavior of injected tumor cells	Long-term analyses are not possible
Possibility to perform xenograft with few tumor cells (100 cells/embryo)	Embryos, after tumor cell implants, have to be raised at a compromise temperature between the optimal for embryos and tumor cells
Tumor-induced angiogenesis within few days from the xenograft (24–48 h post injection)	
Lack of a fully mature immune system in embryos	
Permeability to small molecules	

the digestive system, interrenal gland, thyroid, gonads, fat tissue, kidney, gills and so on. The conservation of the neuroendocrine system is not only at anatomical level (Fig. 1). Indeed, neuropeptides, pituitary hormones and molecular signals from peripheral organs that support the activity of main neuroendocrine axes in zebrafish are very similar to those of mammals and are crucial for maintaining physiological homeostasis. For instance, the organization of the hypothalamic neuroendocrine system of zebrafish is made of nuclei that project into or toward the pituitary as in higher vertebrates (Lohr & Hammerschmidt 2011). Orthologs for six hypothalamic neurohormones that regulate the activity of anterior pituitary gland, such as thyrotropin-releasing hormone, corticotropin-releasing hormone, growth hormone-releasing hormone (GHRH), somatostatin, gonadotropin-releasing hormone and dopamine, have been isolated in zebrafish. Moreover, zebrafish hypothalamus expresses the orthologs of mammalian oxytocin and vasopressin, called isotocin (Unger & Glasgow 2003) and vasotocin (Eaton *et al.* 2008), respectively, that are released into the bloodstream via the posterior pituitary. Like its mammalian counterpart, the zebrafish

pituitary consists of two different parts, which differ in developmental origin and physiology. The posterior pituitary that derives from a ventral extension of the hypothalamus represents the neural compartment of the gland (Pogoda & Hammerschmidt 2007, Toro *et al.* 2009). The anterior pituitary, derived from placodal ectoderm, contains distinct endocrine cell lineages which specifically secrete the thyroid-stimulating hormone, the adrenocorticotropic hormone (ACTH), the α-melanocyte-stimulating hormone, the growth hormone (GH), the follicle-stimulating hormone, the luteinizing hormone, prolactin (PRL) and somatolactin. This last is a member of the GH/PRL family, unique to bony fish, implicated in several physiological processes (energy homeostasis, stress response, reproduction, fat or ion metabolism, acidosis, pigmentation, etc.) (Gonzalez-Nunez *et al.* 2003, Herzog *et al.* 2003, Liu *et al.* 2003, Zhu *et al.* 2004, So *et al.* 2005, Lopez *et al.* 2006, Chen & Chiou 2010, Lohr & Hammerschmidt 2011).

Classical feedback mechanisms, involving signals from peripheral organs, contribute to the regulation of hypothalamic and anterior pituitary hormone secretion. A typical example about the integration of
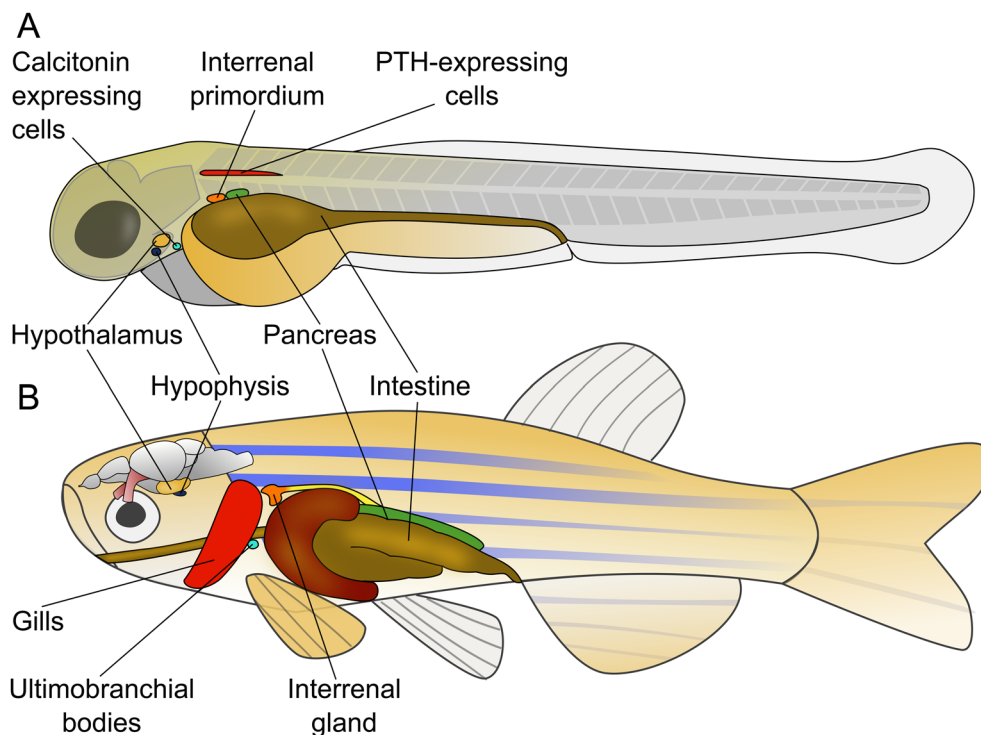

Figure 1
Schematic drawing depicting major zebrafish neuroendocrine structures in a larva of about 3 days post fertilization (A) and in an adult fish (B). Black lines indicate hypothalamus, hypophysis, ultimobranchial bodies and their calcitonin-expressing precursor cells, interrenal gland and its primordium, gills and PTH-expressing cells during larval development and intestine, in which neuroendocrine enterochromaffin cells are dispersed. Pancreas, probably the best characterized endocrine organ, is also indicated.

central and peripheral signals is represented by the hypothalamic-pituitary-interrenal axis that, homologous to the human hypothalamic-pituitary-adrenal axis, regulates the corticosteroid stress response in zebrafish. The hypothalamic CRF stimulates the release of ACTH from the pituitary, which stimulates the secretion of cortisol by the interrenal gland, homologous to the adrenal cortex in mammals. Interestingly, cortisol is the main stress hormone as in humans, while mice and rats utilize corticosterone (Nesan & Vijayan 2013). A negative feedback system acts on the hypothalamus to ensure homeostatic regulation. The stress response in zebrafish is mediated not only by glucocorticoids but also by catecholamine, which are secreted by chromaffin tissue, the homologue of mammalian adrenal medulla (Eto *et al*. 2014).

Moreover, other typical structures of zebrafish neuroendocrine system are conserved compared to human. For instance, zebrafish has calcitonin secreting cells that are homologues to mammalian C-cells. Unlike humans, in which C-cells are dispersed throughout the thyroid parenchyma, these zebrafish cells arise from the ultimobranchial bodies, a bilateral structure close to the heart atrium, which does not fuse with the thyroid (Bourque & Houvras 2011). Calcitonin, secreted by these cells, exerts a hypocalcemic fuction as in the mammalian counterpart (Alt *et al*. 2006).

Gill tissue of zebrafish may represent an evolutionary ancestor of the parathyroid gland in higher vertebrates (Okabe & Graham 2004). Gill cells produce calcium sensing receptor and parathyroid hormone (PTH), whose hypercalcemic function is conserved during the evolution (Lin *et al*. 2014).

Interestingly, zebrafish neuroendocrine system is made not only of anatomically recognizable structures (e.g. pituitary, interrenal gland, etc.), but also of cells that are dispersed in several tissues, similar to the human diffuse neuroendocrine system. For instance, the population of zebrafish enterochromaffin cells in the intestinal tract, as the human counterpart, derives from the neural crest cells and controls intestinal motility by secreting serotonin (Njagi *et al*. 2010).

Moreover, zebrafish has been broadly used to study other endocrine organs. Among these, the pancreas is the most intensively studied. Developmental pathways building and maintaining the cell types of the pancreas are generally conserved in vertebrates. The expression of typical pancreatic hormones, such as insulin, glucagon, somatostatin and ghrelin, has already been detected by 15 h post-fertilization (hpf) in pancreatic progenitor cells

of zebrafish embryos (Tiso *et al*. 2009). Zebrafish adult pancreas shares not only the general anatomical structure with the mammalian pancreas, but also its physiological role in the regulation of glucose metabolism through the secretion of insulin, somatostatin and glucagon (Krishnan & Rohner 2019).

Zebrafish and NETs

Since our previous review (Vitale *et al*. 2014), the number of zebrafish studies on NETs has slightly increased. Below, we summarize recent updates regarding currently available genetic and transplantable zebrafish models for NETs.

Genetic models

Several genetic models, developing NETs or related-syndromes during developmental stages, or in adult zebrafish have been established taking advantage of technologies for the generation of mutant and transgenic animals, as well as for transient modulation of gene expression during embryonic development (Table 2). These models represent a powerful platform to understand carcinogenesis of NETs, as well as to identify new therapeutic strategies.

Between zebrafish mutant lines, there are many noteworthy examples for the study of molecular conditions predisposing to human NETs, even if these zebrafish models do not clearly develop these neoplasms. For instance, inactivating mutations in zebrafish Von Hippel–Lindau (*vhl*) gene led to several key conditions of the human VHL disease, a continuum of multiple endocrine neoplasia (MEN), which is characterized by a constellation of cysts and extensively vascularized tumors, including several NETs such as pheochromocytomas and pancreatic NETs (Richard *et al*. 2013). Although these mutants do not develop NETs, they are characterized by the activation of Hif signaling pathway, severe pathological neovascularization, macular edema, pronephric abnormalities and polycythemia as in human (van Rooijen *et al*. 2011, 2018, 2010). In this frame, *vhl* mutants have been recently used to test the efficacy of several compounds in rescuing VHL phenotype. For instance, it has been demonstrated that sunitinib malate, a multi-tyrosine kinase inhibitor, was able to reverse the ocular, behavioral and morphological phenotypes observed in homozygous *vhl* zebrafish mutants (Ward *et al*. 2019). Therefore, these mutants represent a

Table 2 Currently available zebrafish genetic models for preclinical research in NETs.

	Model	Phenotypes	References
Mutant lines	*vhl* mutants	Partial recapitulation of human VHL phenotype	van Rooijen *et al.* 2010, 2011, 2018, Ward *et al.* 2019
	nf1 mutants	Partial recapitulation to human neurofibromatosis type 1	Shin *et al.* 2012, Ki *et al.* 2017
	tsc2 mutants	Partial recapitulation of human tuberous sclerosis complex phenotype	Kim *et al.* 2011, 2013, Scheldeman *et al.* 2017, Serra *et al.* 2019
	usp39 mutants	Microcephaly and pituitary hyperplasia	Rios *et al.* 2011
	ret mutants	Partial recapitulation of Hirschsprung's disease phenotype	Heanue *et al.* 2016
Transgenic lines	Transient overexpression of human *MYCN* under *myod* promoter	Abdominal tumors resembling human pancreatic neuroendocrine carcinoma	Yang *et al.* 2004
	Transient overexpression of human *MYCN* and *ALK* in peripheral sympathetic nervous system	Tumors resembling human neuroblastoma	Zhu *et al.* 2012
	Stable overexpression of *pttg* under *pomc* promoter	Recapitulation of human Cushing's Disease phenotype	Liu *et al.* 2011
	Stable and ubiquitous overexpression of tilapia GH	Recapitulation of acromegaly phenotype	Elbialy *et al.* 2018
Reverse genetics	*aip* morpholino-mediated knockdown	Hyperplasia of the pituitary gland	Igreja *et al.* 2010, Stojanovic *et al.* 2016
	ret morpholino-mediated knockdown	Partial recapitulation of Hirschsprung's disease phenotype	Burzynski *et al.* 2009

promising platform not only to study molecular basis of VHL disease, but also to identify innovative treatments for this complex pathology.

Another interesting zebrafish mutant model is characterized by Nf1 deficiency (Shin *et al.* 2012), a genetic condition that in humans causes neurofibromatosis type 1. *Nf1* zebrafish mutants have similar phenotypes to those reported in humans, such as abnormal patterning of the melanophores and the predisposition to cancer development, in particular tumors of the CNS or gastrointestinal tract and malignant peripheral nerve sheath tumors (MPNSTs) (Shin *et al.* 2012). Although it has not been reported if these tumors have a neuroendocrine phenotype, zebrafish *nf1* mutants may represent a valid platform to study molecular events underlying tumor susceptibility in patients with neurofibromatosis type 1. Indeed, it has been recently reported that the overexpression of the receptor tyrosine kinase platelet-derived growth factor receptor-α (Pdgfra) in *nf1* mutant background was more active in accelerating MPNST initiation (Ki *et al.* 2017). The kinase inhibitor sunitinib, alone and in combination with the MEK inhibitor trametinib, was able to delay MPNST progression in transgenic fish overexpressing Pdgfra (Ki *et al.* 2017). Interestingly, *nf1* zebrafish mutants are also a promising platform to perform drug screening. In particular, *nf1*

mutants have been used to test the pharmacological inhibition of downstream targets of RAS (PI3K and MAPK) (Ki *et al.* 2017), given that neurofibromin acts as a suppressor of the RAS activity.

Another genetic model with potential applications for the identification of new drugs for NET treatment is the mutant zebrafish line that harbors a nonsense mutation in tuberous sclerosis complex 2 (*tsc2*) gene. Mutations in the human homologous lead to an autosomal dominant disease, characterized by the development of multiple hamartomas and occasionally NETs. Although the occurrence of NETs has not been reported in zebrafish *tsc2* mutants, they exhibited, as TSC patients, hamartoma formation in the brain and activation of the TOR pathway (Kim *et al.* 2011). This pathway has been recently indicated as pivotal for NET tumorigenesis and progression (Manfredi *et al.* 2015). Interestingly, few studies showed the ability of rapamycin, an mTOR inhibitor, in reducing tumor proliferation and vascularization in *tsc2* mutants (Kim *et al.* 2013, Scheldeman *et al.* 2017). Therefore, zebrafish *tsc2* mutant larvae appear to be a potential platform for testing TOR inhibitors (Serra *et al.* 2019) and to identify new therapeutic targets in TSC patients (Scheldeman *et al.* 2017).

Since our previous review, no advances have been reported on the mutant line harboring a mutation in

ubiquitin-specific peptidase 39 (Usp39), a zebrafish model with potential applications in studying a new mechanism for pituitary tumorigenesis (Rios *et al.* 2011).

The generation of transgenic lines is another approach to model NETs in zebrafish. Tumors resembling human pancreatic neuroendocrine carcinoma and human neuroblastoma have been identified in transgenic lines in which human *MYCN* was expressed under zebrafish *myoD* promoter (Yang *et al.* 2004) and in which human *MYCN* and activated anaplastic lymphoma kinase (*ALK*) genes were simultaneously overexpressed in peripheral sympathetic nervous system, respectively (Zhu *et al.* 2012). However, no updates have been recently reported on these models.

Due to the high conservation of main neuroendocrine hormones in vertebrates, transgenesis technology has been used in zebrafish to mimic several conditions associated to functioning NETs, due to excessive release of specific hormones. For instance, the transgenic line that expressed pituitary tumor transforming gene (*pttg*) under the control of proopiomelanocortin (*pomc*) gene in adenohypophyseal cells, showing ACTH-secreting pituitary tumors within the first days of embryonic development and in adult animals, has been proposed as a model for human Cushing Disease, a neuroendocrine disorder due to an uncontrolled ACTH hypersecretion by several NETs (Liu *et al.* 2011). More recently, Elbialy and collaborators established a stable acromegaly transgenic model that ubiquitously and constantly overexpresses GH of tilapia fish (*Oreochromis niloticus*) (Elbialy *et al.* 2018). Acromegaly is a hormonal disorder predominantly caused by a GH-secreting pituitary adenoma and more rarely due to NETs secreting GH or GHRH. Acromegaly patients show acral and facial overgrowth, soft-tissue hypertrophy, cardiovascular diseases, metabolic disturbances, osteoarthritis, an increased incidence of tumors, impaired quality of life and increased mortality (Chanson & Salenave 2008, Fuentes-Fayos *et al.* 2019). Surprisingly, the model of Elbialy recapitulated several aspects of acromegalic patients, such as the acceleration of the growth and a significant increase of insulin-like growth factor I (IGF-I), known to mediate most biological actions of GH. Interestingly, the elevation of the GH/IGF-1 axis in this zebrafish acromegaly model was associated with a significant down-regulation of DNA repair pathways and a robust increase in the number of DNA-damaged cells. These findings provide additional support to explain the increased cancer susceptibility in acromegaly (Elbialy *et al.* 2018). Moreover, this transgenic model may be a reliable

platform to clarify mechanisms by which GH excess induces these complications in acromegalic patients.

Recent studies have also exploited MO technology to knockdown NET-related genes, as in the case of aryl hydrocarbon receptor interacting protein (AIP) gene. The human ortologue is mutated in the germline of about 15–40% of familial pituitary adenomas (Igreja *et al.* 2010), and patients with mutations are predisposed to develop large, invasive, GH- or PRL-secreting pituitary tumors, occurring at a younger age and poorly responsive to treatment (Stojanovic *et al.* 2016). The *aip* knockdown in zebrafish embryos resulted in brain, pericardium and swim bladder anomalies and general developmental delay, suggesting a developmental role. Moreover, morpholino-injected embryos exhibited larger surface of PRL immunostaining in the pituitary compared to controls, suggesting an increase in proliferative activity (hyperplasia or tumour) at pituitary level (Stojanovic *et al.* 2016).

Another peculiar NET-related gene is the *RET* proto-oncogene, whose germline mutations are causative of MEN2, a hereditary disorder characterized by medullary thyroid cancer and other NETs (Vitale *et al.* 2001). The sequence of zebrafish *ret* has a high identity with that of its human orthologue. It has been demonstrated that its MO-mediated knockdown during embryonic development resulted in a complete loss of the zebrafish enteric nervous system (Burzynski *et al.* 2009), as in Hirschsprung's disease, which is associated with human *RET* mutations. A more recent zebrafish model of Hirschsprung's disease, characterized by a point mutation in *ret*, showed that intestinal motility is severely compromised in *ret* homozygous mutants and partially impaired in heterozygous larvae (Heanue *et al.* 2016). Therefore, *ret* mutants, harboring mutations similar to those found in patients with MEN2, could represent a promising platform to study the molecular basis of this disease and to perform drug screening.

NET xenografts in zebrafish embryos

We have described the development of a tumor xenograft model in zebrafish embryos to study NETs, focusing on tumor-induced angiogenesis and invasive behavior of implanted cells (Table 3). The procedure was set up by implanting several immortalized human NET cell lines in the subepidermal cavity of *Tg(fli1a:EGFP)^{y1}* zebrafish embryos, which express EGFP in the entire vascular tree under the control of the endothelial *fli1a* promoter

Table 3 Zebrafish transplantable models for preclinical research in NETs.

| NET implanted cells	Stage	Site of implantation	Applications	References
Immortalized cell lines	48 hpf	Subperidermal cavity	Evaluation of proangiogenic and metastatic behavior, analysis of tumor microenvironment contribute for tumor progression, drug screening of anticancer molecules	(Vitale *et al.* 2014, 2017)
Patient-derived tumor cells	48 hpf	Subperidermal cavity	Evaluation of individual proangiogenic and metastatic behavior, analysis of tumor microenvironment contribution for tumor progression, development of precision medicine	(Gaudenzi *et al.* 2017, Peverelli *et al.* 2017, Wurth *et al.* 2017)

hpf: hours post fertilization.

(Lawson & Weinstein 2002, Vitale *et al.* 2014, 2017). NET grafted cells quickly led to the formation of endothelial structures, sprouting from physiological vessels of the subintestinal vein (SIV) plexus and the common cardinal vein (CCV) within 24 hpi. In the next 48 h, these endothelial sprouts were progressively converted in vessels with heterogeneous diameters that could reach and penetrate the implanted tumor mass (Vitale *et al.* 2014). Tumor-induced angiogenesis is easily and accurately quantified through computerized image analysis. Taking also into consideration the permeability of embryo to small molecules dissolved in the fish water, zebrafish/NET xenograft represents an attractive, fast and technically simple platform to perform drug screening. Moreover, larger or not water-soluble molecules can be injected into the blood stream to ensure drug uptake (Gaudenzi *et al.* 2019). Due to the low proliferation rate of some NETs, the possibility to observe tumor progression in implanted zebrafish embryos in a small temporary window results particularly suitable to test the anti-angiogenic and the anti-metastatic potential of selected drugs, while it may limit the analysis of their anti-proliferative effects.

More recently, we have set up a procedure based on the injection of patient-derived NET tumor cells in zebrafish embryos (Fig. 2 and Table 3) (Gaudenzi *et al.* 2017). The appeal of this model is supported by the growing number of experimental evidences suggesting the use of zPDX in oncological research, substantially for their ability to better mimic the heterogeneity and the behavior of primary tumors compared to immortalized cell lines. In our procedure, NET primary cultures generated from post-surgical samples were stained with a fluorescent dye and implanted into the subperidermal cavity of *Tg(fli1a:EGFP)^{y1}* zebrafish embryos. We have demonstrated that NET zPDXs have a robust proangiogenic potential and a strong invasive behavior. After only 24 hpi, NET cells migrated far from the injection site and invaded different parts of the embryo, in particular the area of the posterior caudal

vein plexus (Fig. 2) (Gaudenzi *et al.* 2017, Peverelli *et al.* 2017, Wurth *et al.* 2017). Interestingly, injected NET cells preserved nuclear morphology and the expression of specific markers (Gaudenzi *et al.* 2017, Peverelli *et al.* 2017, Wurth *et al.* 2017). Due to the possibility to study the effects of small tumor implants (100–1000 cells/embryo), zPDXs resulted particularly suitable for NETs, where the post-surgical availability of tumor cells is often limited (Gaudenzi *et al.* 2017). Moreover, the success of NET transplantation in zebrafish embryos resulted to be extraordinarily higher compared to that reported for PDX murine model (Morton & Houghton 2007). All these results, together with recent evidences about the high potential of zPDX platform in predicting the clinical response to anticancer drugs in colorectal cancer (Fior *et al.* 2017), open a promising scenario for the development of precision medicine applications (Gaudenzi *et al.* 2019). In particular, zPDXs of NETs may be used in co-clinical trials that, up to now, have been developed only in mice. Similar to murine model, patient-derived tumor cells, isolated from a patient enrolled in a clinical trial, may be implanted into zebrafish embryos that are subsequently treated with the same drugs of the patient to emulate clinical response (Byrne *et al.* 2017, Koga & Ochiai 2019). This approach, analyzing and integrating preclinical and clinical data in a real-time manner, could offer the possibility to identify the most appropriate and personalized therapy in patients with NETs, as well as to prevent drug resistance (Table 3).

Moreover, this zebrafish/NET xenograft platform may offer unique opportunities to study the contribution of tumor microenvironment (TME) for tumor progression in NETs. TME is characterized by a complex composition of different cell types including cancer cells, endothelial cells, immune cells and fibroblasts and different molecular players, such as pro-inflammatory and oncogenic mediators. TME is created and shaped by the tumor, which orchestrates molecular and cellular events with the aim to enhance the survival of tumor cells (Wang *et al.* 2017).
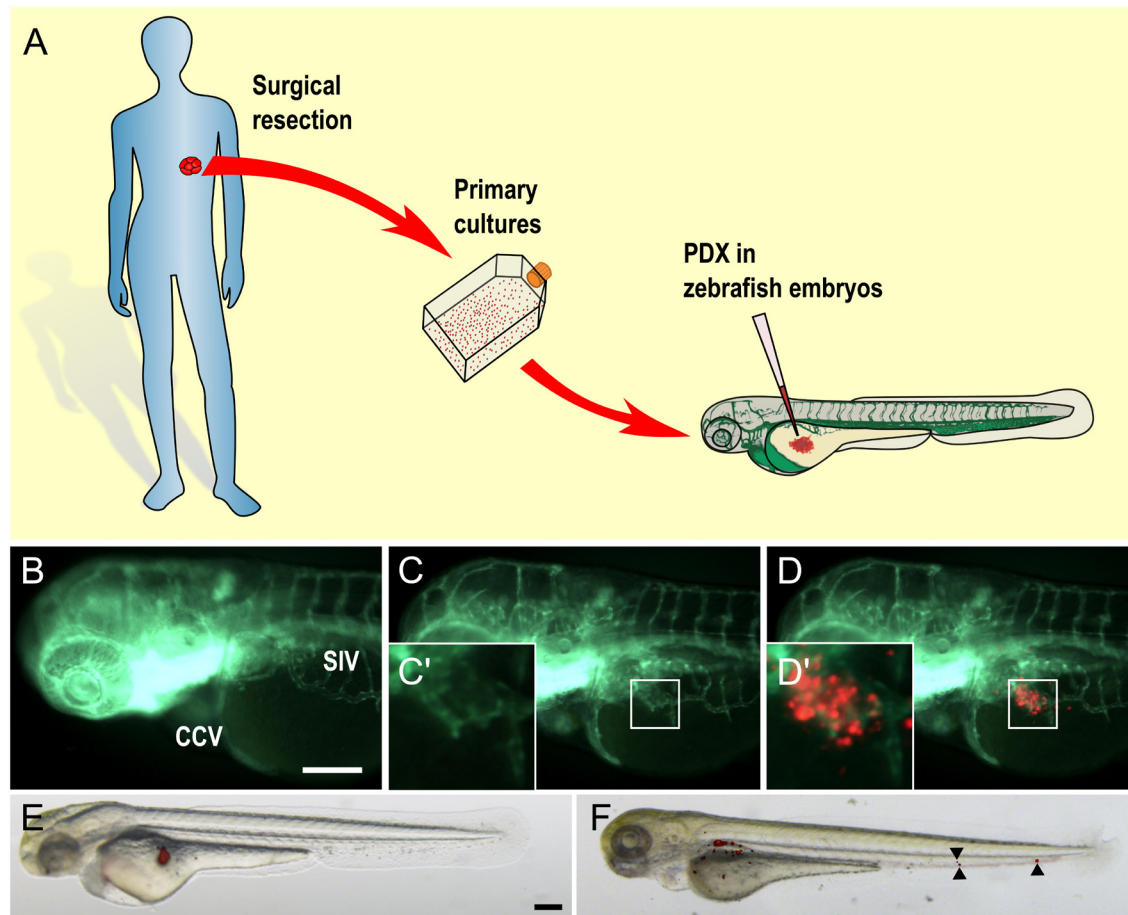
Figure 2

NET-PDX in zebrafish embryos. After the surgical resection, a portion of the fresh tumor is used to establish a NET primary culture. Red stained primary cell suspension is subsequently implanted in 48 hpf *Tg(fli1a:EGFP)^{y1}* zebrafish embryos (A). After the implantation, the pro-angiogenic (B, C and D) and invasive (E and F) potential of patient-derived grafted cells is followed *in vivo*. In this panel, representative epifluorescence and bright-field images, obtained after the implantation of a lung NET, are reported (B, C, D, E and F). Compared to PBS-injected control embryo (B), in which SIV (subintestinal vein) plexus is correctly formed, patient-derived NET xenografted embryo (C–D′) showed the formation of endothelial structures (green), sprouting from the SIV, which reached the implanted tumor mass (red). In C and C′, the red channel was omitted to highlight the newly formed endothelial structures; C′ and D′ are digital magnification of the graft region (white box). Overlay of representative fluorescent and bright-field images of grafted embryos at 0 (E) and 24 hpi (F) showed the spread of NET cells throughout the embryo body. Black arrowheads indicate migrating cells in the area of the posterior caudal vein plexus (F). All images are oriented so that rostral is to the left and dorsal is at the top. Scale bars: 100 µm (B, C, D, E and F).

In this context, zebrafish xenograft is an ideal tool for the observation and analysis of tumor cell cross-talk with key players of TME, with the possibility to recapitulate *in vivo* and in real time its biological heterogeneity. In addition to evaluating tumor-induced angiogenesis, zebrafish have become a powerful model organism to study the innate immune system, mainly because zebrafish larvae have a similar repertoire of innate immune cell lineages to mammals, including neutrophils and macrophages (de Jong & Zon 2005, Keightley *et al.* 2014). In particular, well-characterized reporter lines for imaging and distinguishing different leukocyte behaviors *in vivo* have been generated. These transgenic strains, paired with xenotransplantation of NET cells, may represent a novel tool to analyze the contribution of innate immune cells to the tumor progression in a living selective microenvironment, with significant translational and clinical implications. Different transgenic lines are available, such as *Tg(mpx:EGFP)*, which expresses GFP in neutrophils (Renshaw *et al.* 2006); *Tg(lysC:GFP)* or *Tg(lysC:dsRED)*, whose labeled cells have hallmark traits of myelomonocytic cells, marking a subset of macrophages and likely also neutrophils (Hall *et al.* 2007); *Tg(mpeg1:mCherry)^{gl23}* and *Tg(mpeg1:EGFP)^{gl22}*, which express red or green fluorescent proteins in monocytes/macrophages (Ellett *et al.* 2011). Recently, the fish *Tg(mpeg1:mCherryF/tnfa:eGFP-F)* line, obtained by mating *Tg(mpeg1:mCherry)* fish with a transgenic line whose

macrophages express *tnfa* (tumor necrosis factor alpha), characteristic of classically activated macrophages (M1), allows to show the dynamic macrophage activation in real-time and *in vivo*, including recruitment and phenotypic change after an injury or infection (Nguyen-Chi *et al.* 2015). The use of this transgenic line has emphasized the similarities between zebrafish and human macrophages in terms of diversity and plasticity of macrophage subsets.

Another attractive opportunity will be to create a 'humanized' zebrafish, adopting this fish as an ideal recipient for human neoplastic cells and other components of human TME, trying to reconstitute an interactive microenvironment that recapitulates a clinical situation. This procedure could provide a better understanding of the contribution to tumor progression of each cell type within TME.

It has been recently reported that human macrophages injected into blood circulation and hindbrain parenchyma of living zebrafish embryos can survive and express specific markers, such as TNF-α, CD163 and VEGF, which in part identified M1 and M2 macrophage phenotypes. Moreover, tumor associated macrophages, isolated from different murine and human tumors and co-engrafted with tumor cells in zebrafish embryos, significantly potentiated the capacity of tumor invasiveness and metastasis, in particular M2 respect to M1 (Wang *et al.* 2015, Paul *et al.* 2019).

Finally, zebrafish xenotransplantation model may offer a real-time visualization of the impact of specific pharmacological treatments on TME, with relevant perspectives in the therapy of NETs.

Conclusion and future perspectives

The teleost zebrafish is an experimental model with well-recognized advantages for the study of human tumors, including the heterogenous class of NETs. Although only few zebrafish models developing NETs have been produced until now, the advances in genome sequencing, the molecular conservation of NET-related genes in vertebrates and the availability of techniques to manipulate gene function offer unique opportunities to generate other relevant models in the future. For instance, the conservation of *MEN1 gene* between zebrafish and human may support the identification of zebrafish models to study human multiple endocrine neoplasia type 1 in the future.

The proved conservation of the neuroendocrine system from zebrafish to humans offers the possibility to study in zebrafish the effects of specific hormone

dysregulations, described in human functioning NETs, and provides the development of reliable platforms for drug discovery. A possible experimental approach that may help the study of functioning syndrome could take advantage of transgenic lines that express reporter genes, encoding fluorescent proteins (EGFP, RFP, etc), in hormone producing cells. So far, this approach has been used in endocrine studies, in particular related to pancreatic cells, but could be also adopted for other neuroendocrine cell populations. For instance, Hesselson and collaborators used a transgenic approach to label two distinct populations of β-cells within the developing zebrafish pancreas that originate in distinct pancreatic buds. This transgenic line appeared to be a potential platform to perform drug screening to identify compounds able to regulate β-cell proliferation and function, with potential applications in pathological states that result from their excessive proliferation (e.g. insulinoma) or insufficient β-cell mass (e.g. diabetes mellitus) (Hesselson *et al.* 2009).

New advances in NET research may derived also result from the use of transplantable models, as innovative and promising platforms to investigate molecular events involved in tumor progression, and to perform screening of new anticancer compounds. The reported advantages of NET PDXs in zebrafish embryos, compared to mice, may support the development of precision medicine applications, aimed at predicting the most appropriate and personalized treatment. This approach may represent a breakthrough in the field of NETs, where the clinical management is extremely complex due to the high heterogeneity of these neoplasms in terms of clinical aggressiveness and response to the therapy.

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