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.

Key Words

- neuroendocrine tumors
- zebrafish
- tumor xenograft
- patient-derived xenograft

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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,

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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

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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

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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	

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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

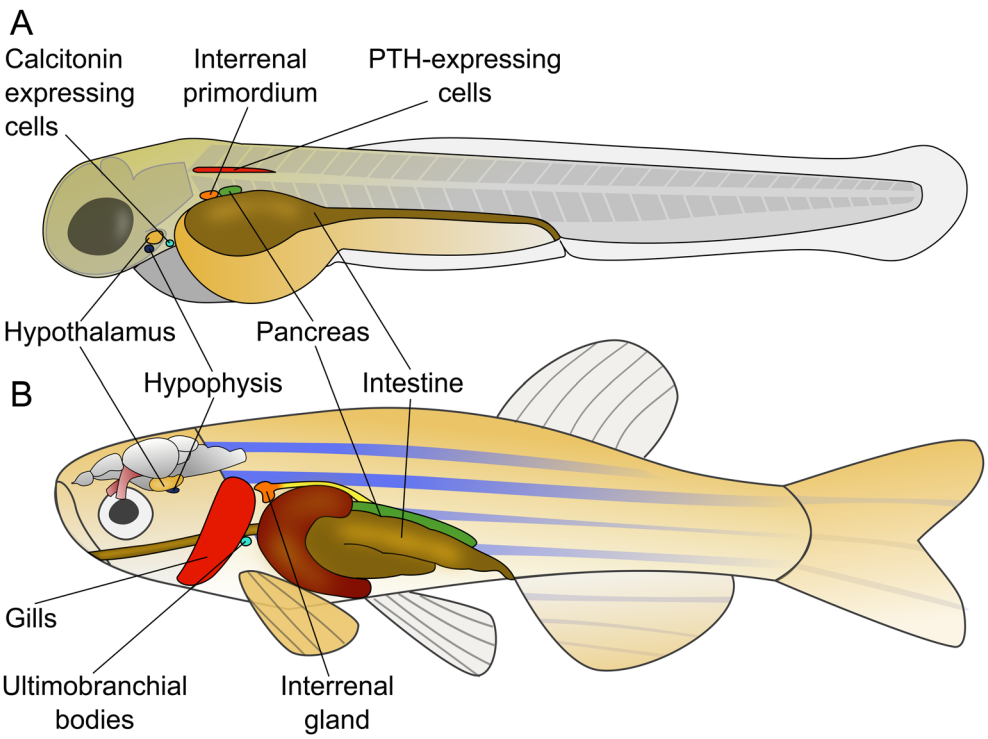

Figure 1
Schematic drawing depicting major zebrafish neuroendocrine structures in a larva of about 3 days post fertilization (A) and in an adult fish (B). Black lines indicate hypothalamus, hypophysis, ultimobranchial bodies and their calcitonin-expressing precursor cells, interrenal gland and its primordium, 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.

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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

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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

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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 subperidermal cavity of *Tg(fli1a:EGFP)*^{y1} zebrafish embryos, which express EGFP in the entire vascular tree under the control of the endothelial *fli1a* promoter

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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).

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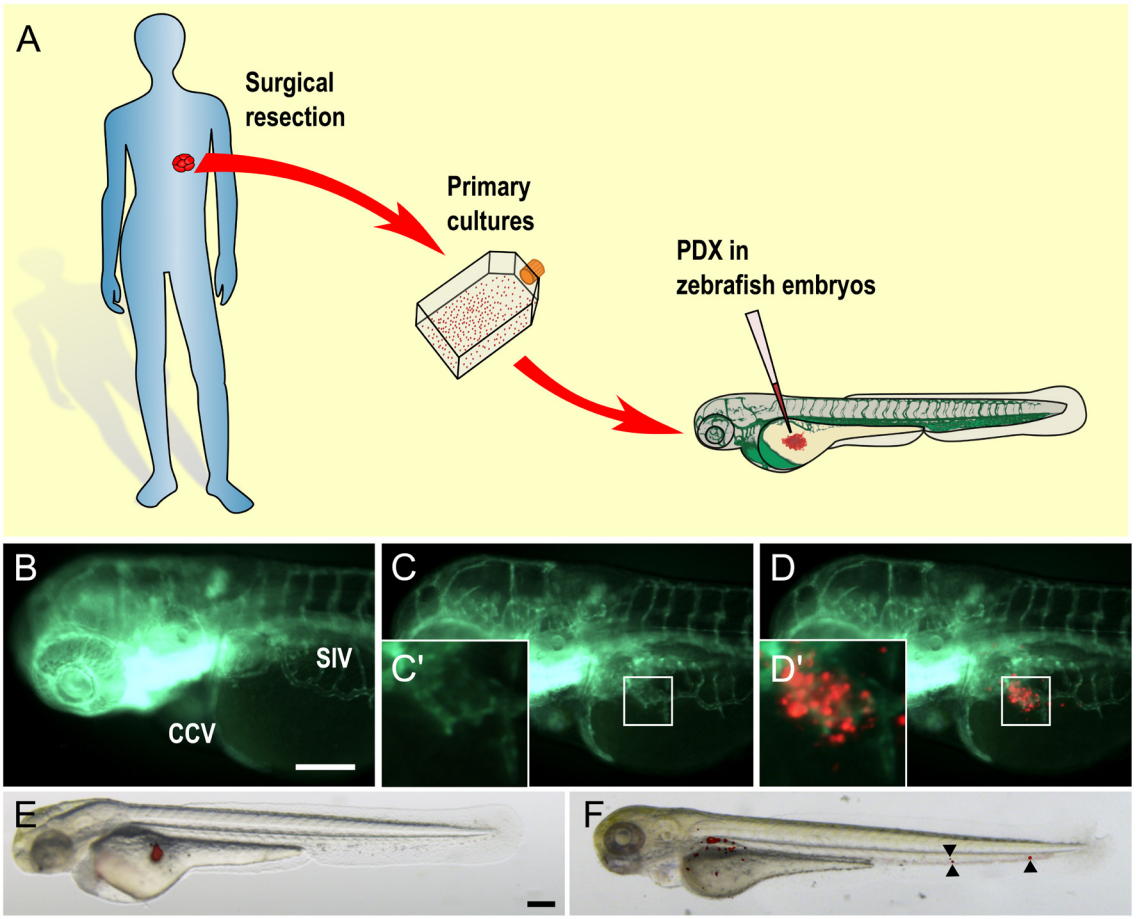

Figure 2
NET-PDX in zebrafish embryos. After the surgical resection, a portion of the fresh tumor is used to establish a NET primary culture. Red stained primary cell suspension is subsequently implanted in 48 hpf *Tg(fli1a:EGFP)*^{y1} zebrafish embryos (A). After the implantation, the pro-angiogenic (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

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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.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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ENDOCRINE METHODS AND TECHNIQUES

Patient-derived xenograft in zebrafish embryos: a new platform for translational research in neuroendocrine tumors

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Abstract Preclinical research on neuroendocrine tumors usually involves immortalized cell lines and few animal models. In the present study we described an in vivo model based on patient-derived xenografts of neuroendocrine tumor cells in zebrafish (*Danio rerio*) embryos, allowing a rapid analysis of the angiogenic and invasive potential. Patient-derived neuroendocrine tumor cells were transplanted in 48 hours post-fertilization *Tg(fli1a:EGFP)*^{y1} zebrafish embryos that express enhanced green fluorescent protein in the entire vasculature. Neuroendocrine tumor cells, stained with CM-Dil, were injected into the subperidermal (perivitelline) space, close to the developing subintestinal venous plexus. A proper control group, represented by zebrafish injected with only D-PBS, was included in this study. Angiogenic and invasive potentials of each patient-derived xenograft were evaluated by both epifluorescence and confocal microscopes. Six out of eight neuroendocrine tumor samples were successfully transplanted in zebrafish embryos. Although the implanted tumor mass had a limited size (about 100 cells for embryos), patient-derived xenografts showed pro-angiogenic (5 cases) and invasive (6 cases) behaviors within 48 hours post injection. Patient-derived xenograft in zebrafish embryos appears to be a reliable in vivo preclinical model for neuroendocrine tumors, tumors with often limited cell availability. The rapidity of this procedure makes our model a promising platform to perform preclinical drug screening and opens a new scenario for personalized treatment in patients with neuroendocrine tumors.

Keywords Neuroendocrine tumors · Zebrafish · Patient-derived xenografts · Angiogenesis

Introduction

Neuroendocrine tumors (NETs) represent a group of rare and heterogeneous neoplasms with a wide spectrum of morphological, functional, and behavioral features [1–3]. At present, only few NET animal models are available [4–6].

The teleost zebrafish (*Danio rerio*) has a relatively complex circulatory system similar to that of mammals and emerged as an attractive human disease model, particularly for cancer research [7].

We have recently developed a novel zebrafish model to investigate tumor angiogenesis in NETs, based on the injection of established human NET cell lines in the proximity of the developing subintestinal vein (SIV) plexus and common cardinal vein (CCV) in zebrafish embryos [8]. This model takes advantage of the intrinsic features of zebrafish embryos (small size, optical transparency, rapid life cycle), as well as of the transgenic *Tg(fli1a:EGFP)*^{y1} zebrafish line that expresses enhanced green fluorescent protein (EGFP) under the control of the *fli1a* promoter, thereby labeling all blood vessels and providing a live

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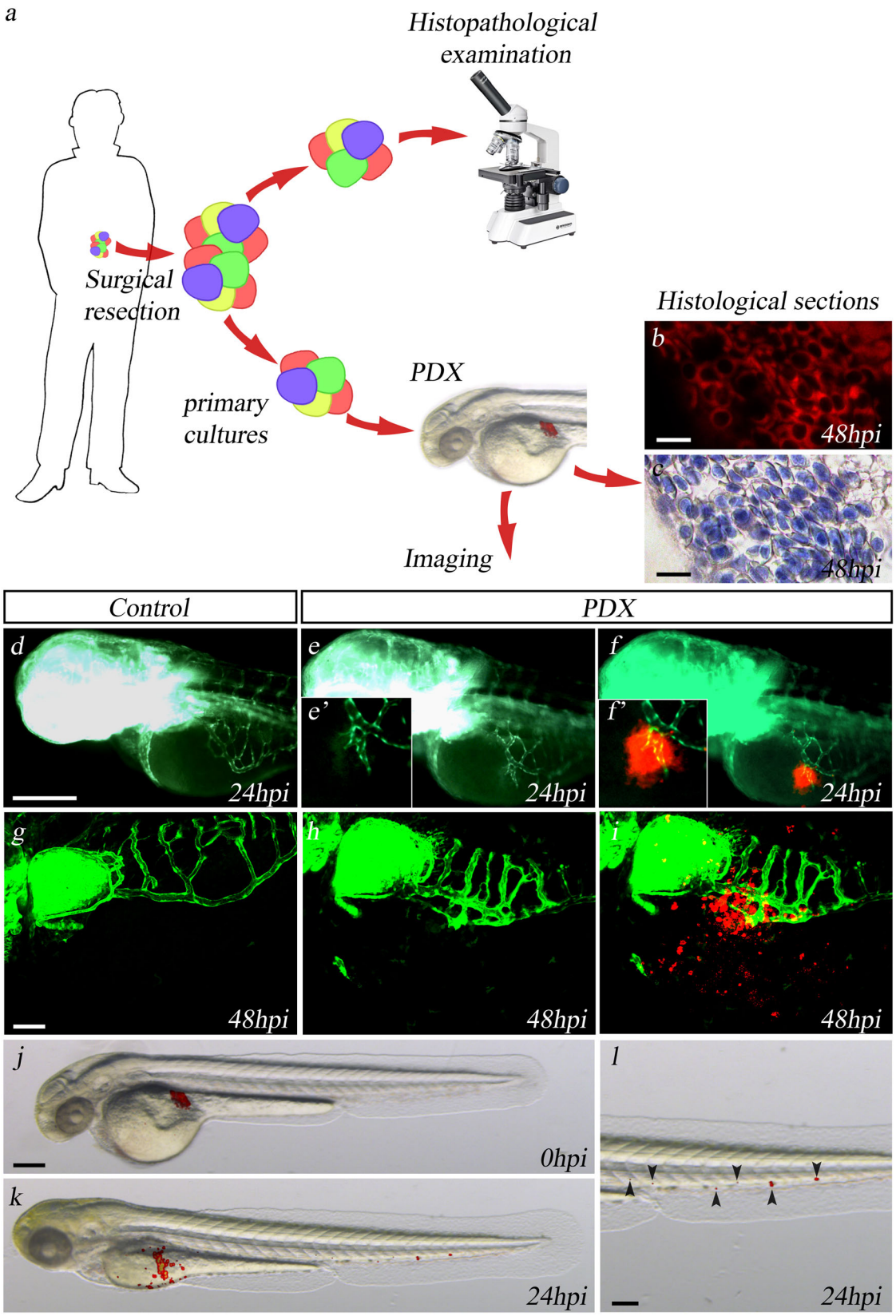
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Histopathological examination
Surgical resection
primary cultures
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Imaging
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◀ **Fig. 1** **a** After the surgical resection, NET sample was evaluated by a pathologist for the routine histopathological examination, while a portion of the fresh tumor specimen was used to establish NET primary culture. Red stained primary cell suspensions were used to perform PDX in zebrafish embryos. In the following 48 hpi, embryos were imaged to monitor the pro-angiogenic and invasive potential. **b** and **c** Transverse histological sections at the PDX level. The hematoxylin staining (**c**) showed that nuclear morphology of red fluorescent NET cells (**b**) was well conserved at 48 hpi. **d–i** Epi-fluorescence images at 24 hpi (**d–f**) and confocal images at 48 hpi (**g–i**) of PBS-injected control embryos (**d**, **g**) and patient-derived NET (red) xenografted embryos (**e**, **f**, **h** and **i**). NET PDX induced the formation of endothelial structures (green) sprouting from the SIV and that reached the implanted tumor mass. In **e** and **h**, the red channel was omitted to highlight the newly formed vessels; **e′** and **f′** are digital magnification of the graft region. **j–l** Overlay of representative fluorescent and bright field images of grafted embryos at 0 (**j**) and 24 hpi (**k**, **l**) showed the spread of NET cells throughout the embryo body. Black *arrowheads* indicate migrating cells in the area of the posterior caudal vein plexus (**l**). All images are oriented so that rostral is to the *left* and dorsal is at the *top*. *Scale bars*: 10 µm (**b** and **c**), 200 µm (**d–f** and **j–l**), 100 µm (**g–i**), 50 µm (**l**)

visual marker for vascular development [9]. NET cell line xenografts, injected between the periderm and the yolk syncytial layer of zebrafish embryos, stimulated the growth of sprouting vessels from the SIV and CCV toward the implant within only 24–72 hours post injection (hpi) [8].

Immortalized tumor cell lines can provide simplified cancer models, however they display a limited ability for predicting anti-cancer drug efficacy in the clinical setting [10]. In the last decade, patient-derived xenograft (PDX) model has emerged as an important tool for translational research, retaining much of the complexity of the tumor microenvironment and heterogeneity of the original tumor in patient [11], and representing the first step toward personalized medicine.

The aim of this paper, is to describe a new in vivo NET model, based on PDX in zebrafish embryos, able to study tumor-induced angiogenesis and cell invasiveness in a time-saving and cost-saving manner.

Material and methods

Zebrafish care

Fish of the strain *Tg(fli1a:EGFP)*^{y1} were raised and maintained according to the National (Italian D.lgs 26/2014) and European (2010/63/EU and 86/609/EEC) animal welfare guidelines. Embryos were collected by natural spawning and staged according to Kimmel and colleagues [12].

Primary culture

NET samples were obtained from the Neurosurgery Division of San Martino Hospital (Genova, Italy) after patients' informed consent and Institutional Ethics Committee approval (number of registry 17/12). Patients were selected according to the availability of tumor specimen for cell culture, after giving priority to the routine histopathological examination (Fig. 1a). Both pituitary adenoma and NET primary cell cultures were obtained from surgical samples as previously described [13, 14]. Tumor cells were purified using anti-fibroblast microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated, viable cells were plated in D-valine Minimum Essential Medium (MEM, PromoCell, Heidelberg, Germany) to suppress the proliferation of any remaining fibroblasts, supplemented with 10 % fetal bovine serum (FBS, Gibco-Thermo Scientific, Milano, Italy), 2 mM glutamine and 1 % penicillin/streptomycin (all purchased from Lonza, Cologne, Germany) at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Procollagen immunofluorescent staining was performed to verify the absence of fibroblast contamination. Cell viability was assessed by trypan blue staining before the injection and it was higher than 90 %.

Tumor xenograft procedure

Twenty-four hours post fertilization (hpf) *Tg(fli1a:EGFP)*^{y1} embryos were soaked in fish water (60 µg/ml instant ocean, 0.1 % methylene blue) with 0.2 mM 1-phenyl 2-thiourea (PTU). After removing the chorion, embryos were incubated for further 24 h at 28 °C. At 48 hpf, embryos were anesthetized with 0.02 mg/ml tricaine and placed on agarose-modified Petri dish, where they were properly oriented with the yolk on a flank. Primary NET cells were stained with the red fluorescent cell tracker CM-Dil (Invitrogen, Life Technologies, Carlsbad, CA, USA) following manufacturer's instructions. In order to prevent cell clumping, cells were re-suspended in D-PBS without calcium and magnesium, added with 0.2 % bovine serum albumin (pH 7.2) and loaded in pre-cooled borosilicate needle [15]. By means of a micro-injector FemtoJet (Eppendorf, Hamburg, Germany), equipped with a micromanipulator InjectMan NI 2 (Eppendorf, Hamburg, Germany), NET cells (about 100 cells per embryo) were grafted into the subperidermal (perivitelline) space, between the periderm and the yolk syncytial layer, in the proximity of the SIV. Correctly grafted embryos were selected on the basis of the xenograft position. At this purpose, we considered only embryos in which injected cells caused a protrusion of the periderm, while embryos in which cells were injected into the yolk sac were discarded. A possible drawback concerning the analysis of migratory behavior for NET cells is represented by the possibility that cells could be erroneously and directly injected into the circulatory system. In order to prevent this pitfall, embryos were observed within 20 min after the injection and those showing cells already outside the yolk

sac region were removed from further analysis. At least 20 correctly grafted embryos were selected for each patient. A proper control group for each PDX, represented by zebrafish injected with only D-PBS, was included in the experimental protocol. All embryos were maintained at 32 °C, a compromise temperature between 28 °C (the optimal temperature for zebrafish maintenance) and 37 °C (the optimal temperature for mammalian cell growth and metabolism). Starting from 24 hpi, both the pro-angiogenic and migratory responses were monitored in vivo by means of both epifluorescence (Leica M205FA equipped with a Leica DFC450C digital camera; Leica, Wetzlar, Germany) and confocal (Nikon C2; Nikon Instruments, Melville, USA) microscopes in all embryos. We considered a positive angiogenic response when the SIV development resulted altered compared to the control and endothelial cells sprouted from the SIV and/or CCV toward the xenograft area. Moreover, we considered as active migration if the labeled NET cells were identified outside the yolk sac region (in the head, trunk and/or tail). At 48 hpi, the angiogenic response and invasive potential of each PDX was reported as the percentage of fish in which we observed angiogenesis and tumor cell migration, respectively. At this stage embryos with injected cells were fixed in 4 % paraformaldehyde, dehydrated, paraffin embedded, and sectioned (6–8 µm). Sections were stained with hematoxylin. The images were acquired with a Leica DM6000 B microscope equipped with LAS Leica imaging software.

Results

Primary NET cell cultures were performed from eights patients. Their clinical and histopathological characteristics are summarized in Table 1. Six out of eight primary cell suspensions were correctly transplanted, whereas in only two cases the engraftment was not successful because of technical problems, such as the limited number of cells and their tendency to form clusters causing needle clogging (Table 1).

Interestingly, NET PDXs showed both pro-angiogenic and invasive behaviors within 48 hpi, and these effects were evident as early as 24 hpi (Figs. 1d–l). While we did not display alterations of the normal vascular developmental pattern in the control embryos, we observed growth of sprouting vessels from the SIV and CCV toward the tumor implant in five out of six PDXs (Figs. 1d–i and Table 1). Moreover, in all six cases NET cells were observed to migrate away from the injection site (Figs. 1j–l and Table 1). Transverse histological sections at the engraftment level showed a well-preserved nuclear morphology of injected NET cells, suggestive of cell viability up to 48 hpi (Figs. 1b, c).

Table 1 Clinical and histopathological characteristics of patients with NETs enrolled to generate PDXs in zebrafish

Patient	Sex	Age (years)	Diagnosis	Ki67 (%)	Stage/tumor size	Hormone hypersecretion	Clinical syndrome	Angiogenesis in PDX (%)	Migration in PDX (%)
1	M	72	Ileal NET, G2	11	IV	—	—	failed xenotransplantation	failed xenotransplantation
2	M	64	Liver metastasis from ileal NET, G1	1	IV	Serotonin (5-HIAA)	Carcinoid syndrome	33	67
3	F	49	Non-functioning pituitary adenoma	0.9	Macroadenoma	—	—	50	33
4	F	39	Non-functioning pituitary adenoma	1	Macroadenoma	—	—	33	67
5	M	74	Non-functioning pituitary adenoma	0.8	Macroadenoma	—	—	25	45
6	F	70	ACTH-secreting pituitary adenoma	1	Macroadenoma	ACTH	Cushing's syndrome	0	33
7	M	75	Liver metastasis from pancreatic NET, G1	1	IV	Calcitonin	—	50	88
8	M	40	ACTH-secreting pituitary adenoma	0.2	Macroadenoma	ACTH	Cushing's syndrome	failed xenotransplantation	failed xenotransplantation

The angiogenic and invasive potential of each PDX was reported as the percentage of fish in which we observed tumor-induced angiogenesis and tumor cell migration at 48 hpi

M male, *F* female, — absent

Discussion

When compared to other vertebrate model systems, zebrafish embryos offer several advantages. The short generation time, the large number of offspring, the transparency (enabling noninvasive imaging), the external development of the embryos and the small size make zebrafish a more practical and less expensive laboratory system than other in vivo cancer models [16]. Interestingly, the zebrafish model provides unique tools for visualization of tumor cell behavior and interaction with host cells. The easy and rapid visualization of the tumor-induced angiogenesis makes zebrafish/tumor xenograft model a powerful tool to investigate molecular events involved in angiogenesis. The appeal of zebrafish xenograft lies also in the possibility to overcome some drawbacks of murine xenograft, such as the large number of tumor cells needed (about 1 million), the long time required (from several weeks to months) to have a visible tumor implant, the need of immunosuppressed animals to avoid transplant rejection and high difficulties to generate mouse xenotransplant models able to metastasize [17]. A very limited number of grafted tumor cells in zebrafish embryos can stimulate angiogenesis within few days and without the need of immunosuppression, because the adaptive immune response is not completely developed during the first month of zebrafish life [18, 19]. Moreover, the use of fluorescent tumor cells provides to investigate their invasive behavior after transplantation into zebrafish embryos. The transparency of the zebrafish embryos allows to follow the very early steps of invasion, circulation of tumor cells in blood vessels, colonization at secondary organ sites and metastasis formation in real-time. This is an aspect that, to date, cannot be investigated in established mouse tumor models [19–21].

In this paper, we describe a promising method for the NET preclinical research, based on the injection of patient-derived tumor cells into the subperidermal cavity of *Tg (fli1a:EGFP)*^{y1} zebrafish embryos. The proposed modality of injection allowed us to study two relevant aspects of tumor progression, such as tumor angiogenesis and invasiveness. The procedure is technically simple, and the possibility to study the effects of small tumor implants (100 cells/embryo) makes this model particularly suitable for NETs, where tumor cells availability is often limited, because of the small size of available tumor samples for research purpose. Taking into consideration the rarity and the low aggressiveness of implanted tumors, the success of transplantation in zebrafish embryos resulted to be extraordinary higher (75 %) compared to that reported for PDX murine model [22, 23]. Indeed, we show that in only two out of eight cases (25 %) few technical problems compromised the success of transplantation. In this study, we used a very heterogeneous NET series, suggesting the applicability of PDX to this wide class of tumors. In the preclinical research, the proposed method could be used to evaluate the correlation between several NET parameters (Ki67, tumor markers, production of hormones, etc.) and both angiogenetic and metastatic behavior. Moreover, NET PDX in zebrafish embryos may represent a promising platform to perform preclinical drug screening, before moving on to the more costly and time-consuming murine model. Because of the permeability of zebrafish embryos to small molecules, a number of compounds can be added directly to the embryo water, whereas larger or not water-soluble molecules can be injected into the body of the embryo to ensure drug uptake. Several publications have already demonstrated that few days after the injection of tumor cells are enough to test the effects of drugs on the peritumoral vascular density in zebrafish embryos [24, 25]. This model could be also useful to predict drug sensitivity in patients, opening a new scenario for the most appropriate and personalized treatment. In conclusion, this preliminary study suggests the applicability of a new zebrafish/PDX model as an innovative platform to investigate molecular events involved in tumor angiogenesis and migration in NETs. Future studies with a larger sample size should focus on investigating its potential utility in the therapeutic decision-making of NETs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Phenotypical and Pharmacological Characterization of Stem-Like Cells in Human Pituitary Adenomas

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Abstract The presence and functional role of tumor stem cells in benign tumors, and in human pituitary adenomas in particular, is a debated issue that still lacks a definitive formal demonstration. Fifty-six surgical specimens of human pituitary adenomas were processed to establish tumor stem-like cultures by selection and expansion in stem cell-permissive medium or isolating CD133-expressing cells. Phenotypic and functional characterization of these cells was performed (1) ex vivo, by immunohistochemistry analysis on paraffin-embedded tissues; (2) in vitro, attesting marker expression, proliferation, self-renewal, differentiation, and drug sensitivity; and (3) in vivo, using a zebrafish model. Within pituitary adenomas, we identified rare cell populations expressing stem cell markers but not pituitary hormones; we isolated and expanded in vitro these cells, obtaining fibroblast-free, stem-like cultures from 38 pituitary adenoma samples. These cells grow as spheroids, express stem cell markers (Oct4, Sox2, CD133, and nestin), show sustained in vitro proliferation as compared to primary cultures of differentiated pituitary adenoma cells, and are able to differentiate in hormone-expressing pituitary cells. Besides, pituisphere cells, apparently not tumorigenic in mice, engrafted in zebrafish embryos, inducing pro-angiogenic and invasive responses. Finally, pituitary adenoma stem-like cells express regulatory pituitary receptors (D2R, SSTR2, and SSTR5), whose activation by a dopamine/somatostatin chimeric agonist exerts anti-proliferative effects. In conclusion, we provide evidence that human pituitary adenomas contain a subpopulation fulfilling biological and phenotypical signatures of tumor stem cells that may represent novel therapeutic targets for therapy-resistant tumors.

Keywords Pituitary adenoma · Cancer stem cells · Cell proliferation · Spherogenesis · Somatostatin receptors

Roberto Würth and Federica Barbieri contributed equally to this work.

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Introduction

Cancer stem cell (CSC) theory radically changes the traditional view of cancerogenesis: at odds with the stochastic hypothesis, in which all cells within the tumor mass retain the same tumorigenic potential, a hierarchical model was developed [1, 2], proposing that tumors originate from rare cells endowed with stem cell-like properties, named CSCs or tumor-initiating cells. CSCs maintain themselves via asymmetric division (self-renewal), from which also the bulk of "differentiated"

cells forming the tumor mass is generated. Accordingly, tumors can develop and grow only when CSCs are preserved. This view also modified the pharmacological approach to tumors since persistence of CSCs, even after apparent tumor eradication, will inevitably result in cancer relapse. Additionally, cytotoxic drugs are scarcely effective on CSCs, which are usually slow-cycling and overexpress DNA repair enzymes and drug-extruding ATPases. CSCs, initially identified in leukemia [3] and in aggressive solid tumors (breast cancer and glioblastoma) [1, 4], are now proposed to be at the basis of all malignancies.

More controversial are the presence and the functional role of stem-like cells in benign tumors, and, in particular, in human pituitary adenomas (hPAs). Although considered a rare neoplasia, hPA prevalence in autopsies may reach 25 % [5]. About 20 % of adenomas secrete growth hormone (GH) causing acromegaly, while hypersecretion of prolactin (PRL) is much more common representing the main cause of infertility in women. Tumors co-secreting GH and PRL are also rather frequent, while hypersecretion of adrenocorticotropin and thyrotropin-secreting adenomas, responsible for Cushing's disease and secondary hyperthyroidism, respectively, are less common. In a significant percentage of cases, hPAs are classified as clinically non-functioning (NFPAs), being either true non-secreting tumors, or releasing inactive proteins (glycoproteic hormones α-subunit), or full gonadotropins, but are not cause of clinical endocrine disorders. NFPAs, but also large hormone-secreting tumors, are often diagnosed for local mass effects, causing headaches, visual alterations, due to optic nerve compression, and hypopituitarism. Thus, mass-related effects and hormone hypersecretion syndromes induce high morbidity and mortality in hPA patients [6].

Adult pituitary needs high plasticity to govern the continuously changing hormonal requirements (e.g., the increases in lactotropes during pregnancy and lactation), as well as its homeostatic cell turnover [7]. On these premises, it was proposed that adult stem cells might control the generation of cells with a specific hormonal phenotype according to the physiological requirements [8]. Stem cells were indeed identified in adult pituitary [8–13], although significant differences were reported as far as the origin and phenotypical characterization are concerned [14], so that they might represent heterogeneous groups of cells [15, 16]. This evidence suggests that hPA might originate from adult pituitary stem/progenitor cells [9]. For example, the expression of the chemokine receptor CXCR4, considered a stem cell signature in different tissues, including pituitary [8], is expressed in subsets of cells of human normal pituitary (mainly hormone-secreting, but also hormone-negative cells) [17, 18]. However, due to the proliferative activity induced by CXCR4 in different human tumors [19, 20], and CXCR4 being co-expressed with its ligand CXCL12 in 100 % of hPA cells, it was proposed that adenomas might derive from the expansion of CXCR4-expressing cells, gaining proliferative advantage from the autocrine/paracrine secretion of CXCL12 [17, 21], resulting in the activation of multiple intracellular mechanisms [22, 23]. GFRA2, another proposed marker of normal pituitary stem cells (GPS cells, determined by the expression of GFRA2 and Prop1 stem markers), is overexpressed in NFPAs, but downregulated in GHomas [24]. However, although suggestive, these studies did not demonstrate the direct derivation of adenoma cells from adult pituitary stem cells and, more importantly, did not show a stem-like activity of hPA cells.

Few subsequent studies reported the isolation of cells with stem-like features from human GHomas and NFPAs (hPA stem cells, hPASCs), but no conclusive results were reached. Analyzing cells isolated from two adenomas, sphere-forming cells (an in vitro index of self-renewal [25]) expressing CD133 and nestin [26] were identified. When grown in the presence of serum, these cells acquire neural (β-III tubulin) and glial (GFAP, S100) phenotypes and released GH and follicle-stimulating/luteinizing hormones after stimulation with growth hormone-releasing or luteinizing hormone-releasing hormone. On the other hand, in response to the respective releasing peptides, also spheroid cells secreted PRL and thyrotropin. Spheroid cells were tumorigenic after intracerebral graft in immunodeficient mice, in vivo tumorigenicity being a required feature to define bona fide tumor stem-like cells. However, the low number of hPA analyzed, the expression of neuronal markers after differentiation, and the nonspecific pituitary hormone release were critical issues in this study. Another report identified a spherogenic $CD133^+$ cell subpopulation in 20 hPAs, showing, for only one culture, a modest tumorigenic activity when ectopically (subcutaneously) implanted in mice [27]. In another study, progenitor mesenchymal cells were also isolated from three GHomas and three NFPAs, but these cells lacked the expression of pituitary-related markers [28]. Finally, sphere-initiating cells were identified in a purified side population isolated from hPAs, overexpressing Sox2, but they were not tumorigenic in mice [29].

Stem-like cells have also been isolated in murine models of pituitary adenomas. In $Rb^{+/-}$ mice, spontaneously developing pituitary tumors, putative stem cells were isolated for Sca1 expression [30]. These cells grow as spheroids, express adult pituitary stem markers (Sox2, nestin, and CD133), and are tumorigenic. Similar results were obtained in dopamine receptor 2 (D2R) knockout mice developing prolactinomas which contained a higher number of side population (putative stem cells), $Sox2^+$, and colony-forming cells than wild-type pituitaries [29].

Thus, published data on the possibility that the tumor stem-like cell paradigm can be extended to hPAs are still incomplete and divergent on several aspects.

With the aim of providing a phenotypical and biological characterization of hPASCs, using postsurgical specimens, here we report: (1) the presence in hPA sections of rare cells

expressing stem cell markers, but not pituitary hormones; (2) the isolation and expansion in vitro of putative hPASCs, phenotypical characterization, and the assessment of spherogenic activity; (3) the sustained in vitro proliferation and survival of the stem-like cells, as compared to the short-term growth of differentiated hPA cells; (4) the ability of this subpopulation to differentiate in vitro into hormone-expressing pituitary cells; (5) the tumorigenic, pro-angiogenic, and highly migratory ability of these cells when injected in zebrafish embryos; and (6) the expression of regulatory pituitary receptors (D2R and somatostatin receptors 2 and 5, SSTR2 and SSTR5) in hPASCs and the inhibition of their proliferation by dopamine/somatostatin bispecific agonists.

Materials and Methods

Tissues and Primary Cultures from Human Pituitary Adenomas

Samples were obtained from the Neurosurgery IRCCS-AOU San Martino-IST Genova (2009–2015), after patients' informed consent and Institutional Ethical Committee approval (Table S1). Histological sections from four autoptic normal pituitaries (Table S2) were also analyzed.

Fifty-six unselected surgical specimens of human pituitary adenoma (15 GHomas, 3 mixed GH-PRL, 1 ACTHoma, and 37 NFPAs) were entered in the study. After surgery, the samples were divided into one portion fixed in formalin, paraffin-embedded, and processed for histopathology and immunohistochemistry and a second fragment that was immediately processed for the primary culture's establishment. Due to the limited availability of adenoma tissue, not all of the experimental approaches described in this study were performed in all the tumors collected (see Table S1). Immediately after reaching the laboratory, the samples were mechanically dissociated under sterile conditions to obtain single cell suspensions. Pituitary cells were purified from fibroblasts using columns loaded with anti-fibroblast microbeads (Miltenyi Biotec, Cologne, Germany). Purified cells were seeded in two different media: (a) standard medium, containing Minimum Essential Medium (MEM) added with D-valine to avoid fibroblast proliferation, supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, and 1 % penicillin–streptomycin (Lonza) [31], and (b) stem cell-permissive medium, containing MEM/HAM'S F12 (1:1, Lonza) supplemented with 1 % FBS, 2 mM L-glutamine, 1 % penicillin–streptomycin, B27 (50X, Life Technologies), 10 ng/ml leukemia inhibitory factor (LIF, Sigma-Aldrich), 20 ng/ml basic fribroblast growth factor (bFGF), and 20 ng/ml epidermal growth factor (EGF; Miltenyi Biotec) [32].

$CD133^+$ Cell Sorting

Dispersed human primary pituitary adenoma cells were grown for 1 day in 10 % FBS-containing medium and then sorted for CD133 expression with MACS microbeads (Miltenyi Biotec). Briefly, cells were dissociated and resuspended in phosphate-buffered saline (PBS), 0.5 % bovine serum albumin, and 2 mM EDTA, and anti-CD133 microbeads were used for positive magnetic cell separation. After three washes, both positive and negative fractions were collected, centrifuged, counted, and resuspended in the respective media. $CD133^+$ cells were then cultured in stem cell-permissive medium for 1 week before immunocytofluorescence (ICF) is performed.

Immunohistochemistry and Double Immunohistofluorescence

Immunohistochemistry (IHC) and immunohistofluorescence (IHF) were performed on 4-μm sections of formalin-fixed paraffin-embedded tissue stained with the primary antibodies reported in Table S3. As reported [33], deparaffinized/rehydrated sections were incubated in sodium citrate buffer (pH 6) for antigen retrieval and rinsed in Tris-buffered saline. Nonspecific immunoreactivity was blocked with 10 % normal goat serum (NGS, Sigma-Aldrich) and primary antibodies applied overnight at 4 °C. IHC was performed using the EnVision™+ Dual Link System-HRP (Dako) following the manufacturer's instructions. For IHF staining [34], sections were labeled with 1:200 fluorochrome-conjugated goat anti-mouse and anti-rabbit secondary antibodies. Negative controls, omitting primary antibodies, were included in all the experiments, adding isotype immunoglobulins for each primary antibody (Fig. S1). For double IHF, mixtures of the primary antibodies and mixtures of the species-specific secondary antibodies were applied simultaneously to sections. Slides were visualized and photographed with a DM2500 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a DFC350FX digital camera (Leica). Co-localization was analyzed by confocal laser scanning microscopy (Bio-Rad-MRC 1024 ES) and the LaserPix software (Bio-Rad).

Immunocytofluorescence

Cells were seeded onto glass coverslips ($d = 12$ mm, 5,000 cells/coverslip) or chamber slides (BD Falcon) 2 days prior to staining, washed twice with PBS, fixed for 15 min at room temperature with 4 % paraformaldehyde, and permeabilized for 4 min in PBS containing 0.1 % (*v*/*v*) Triton X-100. Cells were then blocked for 30 min in PBS plus 10 % NGS and stained with appropriate antibodies. Nuclei were stained with DAPI at room temperature for 5 min and the coverslips mounted in Mowiol-DABCO (Sigma-Aldrich). Immunofluorescence detection was performed as described

for IHF in the previous paragraph. Negative controls, omitting primary antibodies, were included in all the experiments (Fig. S1).

Sphere Formation Assay

Primary spheres were obtained by cells seeded at low density (1000 cells/well) in stem cell-permissive medium. To demonstrate the persistence of self-renewal after long-lasting in vitro culturing, primary spheres were dissociated and isolated cells allowed to generate secondary spheres [35]. In selected cases, sphere-forming efficiency (SFE) was calculated as the percentage of spheres generated over the number of plated cells (100 cells/well).

Cell Proliferation Assay

As an index of cell number, mitochondrial activity was evaluated by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) [36].

In Vivo Models to Assess the Tumorigenicity of Human Pituitary Adenoma Stem-Like Cells

Mouse Model

Animals NOD-SCID mice (6–8 weeks old) were purchased from Charles River (Calco, Italy) and housed in IRCCS-AOU San Martino-IST animal facility.

Tumor Xenograft Pituitary adenoma cell tumorigenicity was assessed, after IRCCS-AOU San Martino-IST (Genova, Italy) Institutional Animal Care and Use Committee (IACUC) approval, by xenografting tumor cells grown in stem cell-permissive medium. Two mice for each adenoma cell culture were used. Mice were anesthetized with 20 μl i.m. of ketamine (2 %) and xylazine (100 mg/ml) and either injected in the flank with 10 or 20×10^5 cells ($n = 2$ adenoma cultures) with Matrigel (BD Biosciences) or positioned into a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). A hole was made in the skull using a 21-gauge needle, 2.5 mm lateral and 1 mm anterior from the intersection of the coronal and sagittal sutures (bregma), and 50×10^5 cells were injected in the left corpus striatum using a Hamilton syringe (series 7000; Sigma-Aldrich) at a depth of 3.5 mm (total volume, 2 μl) [37] ($n = 4$ adenoma cultures). Mice were monitored for more than 8 months for disease symptoms and killed by CO_2 asphyxiation when they showed weight loss or any sign of disease.

Zebrafish Model

Animals Breeding fish of the Tg(*fli1*:EGFP)^{y1} transgenic line were maintained at 28 °C on a 14-h light/10-h dark cycle. Embryos were collected by natural spawning [38], staged as reported, and raised at 28 °C in fish water (Instant Ocean, 0.1 % methylene blue) in Petri dishes, according to National (Italian D.lgs 26/2014) and European laws (2010/63/EU and 86/609/EEC).

Tumor Xenograft Dechorionated embryos at 48 h post-fertilization (hpf) were anesthetized with 0.04 mg/ml of tricaine (Sigma-Aldrich). Pituitary adenoma stem cells (0.5×10^3/embryo) labeled with a red fluorescent dye for cell viability (CellTracker™ CM-DiI, Invitrogen) and resuspended in PBS were implanted in the sub-peridermal space, close to the sub-intestinal vessels (SIV) plexus, of 48 hpf Tg(*fli1*:EGFP)^{y1} zebrafish embryos, as previously reported [39, 40]. To assess that the injection of cell suspension was in the correct region of the embryo, fishes were observed 2 h after the injection and those showing cells into the yolk sac or in the vasculature were excluded from further analysis, while correctly grafted embryos were incubated at 32 °C. A control group, represented by zebrafish injected with PBS only, was included.

The presence of circulating grafted cells along the body, the formation of micrometastasis, and the vasoproliferative response triggered by the tumor xenografts were evaluated through a fluorescence stereomicroscope (Leica DM6000B equipped with LAS Leica imaging software). The spread throughout the embryo body was quantified by Fiji software comparing the area occupied by tumor cells soon after the implant and at 24 h after injection (hpi) [41]. At least 20 correctly grafted embryos were analyzed in independent experiments.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted with RNAse-free DNAse I using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories) and reverse transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad). Single-stranded cDNA products were analyzed by real-time polymerase chain reaction (PCR) using the SsoFastTM EvaGreen mix on a CFX96 Touch real-time PCR (Bio-Rad). Cycling conditions were set at 98 °C for 5 s and 60 °C for 10 s for 40 cycles. Primer sequences were:

Oct4: forward: 5′-CTTCGCAAGCCCTCATTTCAC-3′; reverse: 5′-GAAGGCGAAATCCGAAGCCA-3′
Sox2: forward: 5′-CAGGAGTTGTCAAGGCAGAGA-3′; reverse: 5′-GTCCTAGTCTTAAAGAGGCAGCA-3′

Nestin: forward: 5′-TGGCTCAGAGGAAGAGTCTGA-3′; reverse: 5′-TCCCCCATTCACATGCTGTGA-3′
NANOG: forward: 5′-GTCCCAAAGGCAAACAACCC-3′; reverse: 5′-TTGACCGGGACCTTGTCTTC-3′
CD133: forward: 5′-GCCACCGCTCTAGATACTGC-3′; reverse: 5′-GCTTTTCCTATGCCAAACCA-3′
CXCR4: forward: 5′-AACCAGCGGTTACCATGGAG-3′; reverse: 5′-CTTCATGGAGTCATAGTCCCCTG-3′
28S: forward: 5′-CCCAGTGCTCTGAATGTCAA-3′; reverse: 5′-AGTGGGAATCTCGTTCATCC-3′
GAPDH: forward: 5′-ACCCACTCCTCCACCTTTGA-3′; reverse: 5′-CTGTTGCTGTAGCCAAATTCGT-3′

Levels of target genes in each sample were normalized on GAPDH and 28S amplification and reported as relative values. To confirm specificity of amplification, PCR products from each primer pair were subjected to melting curve analysis.

Statistical Analysis

All experiments were performed at least in triplicate, and the mean ± SEM was plotted. Comparison among multiple groups was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's post hoc tests. Two-tailed *t* tests were used for comparisons between two groups after performing normality test by D,Agostino and Pearson's test, using GraphPad Prism (GraphPad Software, San Diego, USA). Statistical significance was established at $p < 0.05$.

Results

Expression of Stem/Progenitor Cell Markers in hPA Sections

To identify putative hPASCs, 12 hPA sections (seven GHomas and five NFPAs) were analyzed by IHF and IHC for Oct4, Sox2, CD133, nestin, and NANOG. Although almost all of the hPAs showed groups of cells expressing these proteins, the expected heterogeneous expression pattern was identified within the sections, with positive cells mainly grouped in small areas or identified as isolated cells (Fig. 1a and Fig. S2 depict representative images). In detail, we identified Sox2- and CD133-expressing cells in 100 % of the hPA, and nestin and Oct4 in about 90 %. Conversely, NANOG was detected in a smaller percentage of cases. However, since positive cells are non-homogeneously distributed within the adenoma and the impossibility to analyze serial sections for each sample, the occurrence of false negative results cannot be excluded.

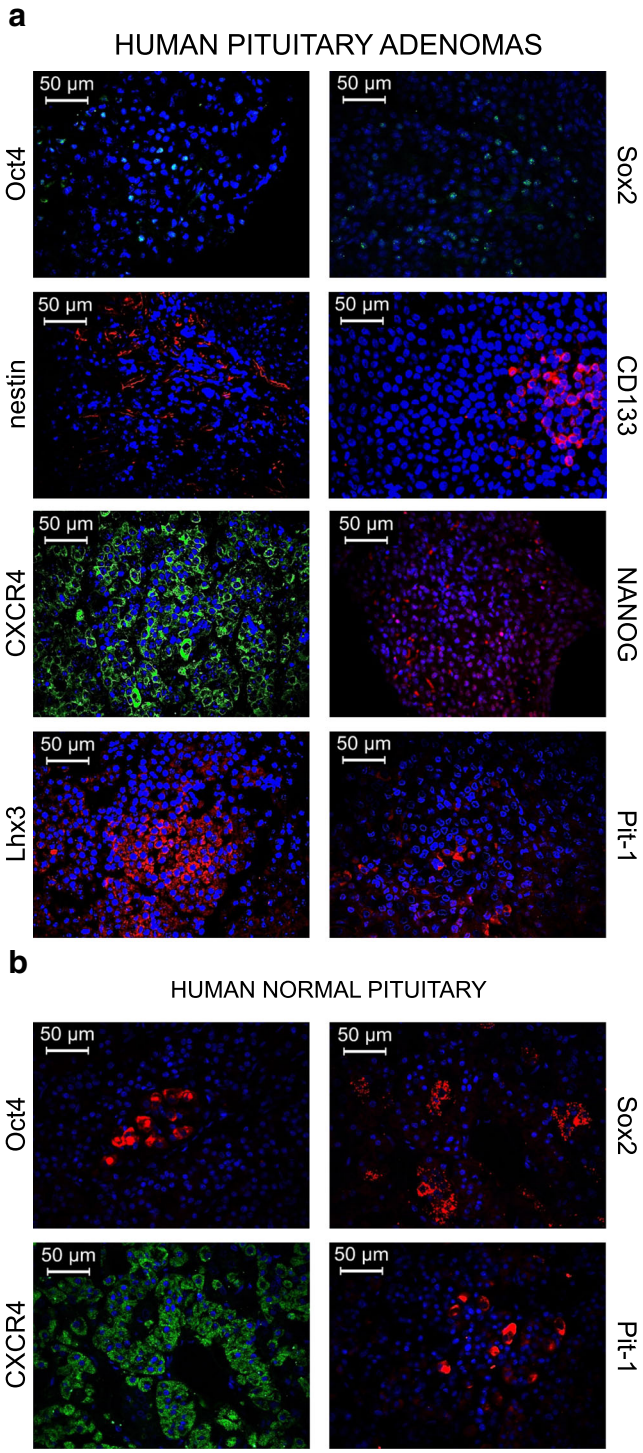

Fig. 1 Immunohistofluorescence analysis for stem cell markers in pituitary adenoma (**a**) and normal pituitary (**b**) tissues. Representative immunofluorescence showing that stem cell marker expression is restricted to limited areas or rare cells. Only CXCR4 is homogenously expressed by pituitary adenoma cells and in large areas of normal pituitary. Nuclei counterstaining was performed with DAPI. Individual adenoma marker expression is reported in Table 1. Negative controls are reported in Fig. S1A (magnification, ×40)

In normal human pituitaries ($n = 4$; Fig. 1b and Fig. S2), the expression of relevant stem cell markers was less evident:

Sox2 and Oct4 were detected in isolated cells, but nestin was negative in almost all samples, as reported [27, 42]. This observation highlights a difference between human and murine normal pituitary since, in the latter species, several studies identified nestin$^+$ stem-like cells [9, 43, 44]. In contrast to hPA in which we observed a preferential Sox2 and Oct4 nuclear localization likely representing a constitutive activation state (Fig. 1a), in normal pituitary, these transcription factors were mainly cytosolic (Fig. 1b), as previously reported within the putative niche (the anterior pituitary marginal zone) [9]. In agreement with previous studies [17, 45], CXCR4 was detected in all hPAs analyzed, labeling all tumor cells. This observation does not confirm previous data in which CXCR4 expression was related to the invasive behavior of pituitary adenomas [46]. In fact, although we did not perform a quantitative analysis, CXCR4 expression was detected in all the cells of all hPAs tested, irrespective of their clinical behavior. Interestingly, the pituitary-specific transcription factors, Pit-1 and Lhx3, identified in pituitary cells committed to differentiation lineages [47], were expressed with high frequency (73 and 80 %, respectively) as rare cells within both GHomas (Pit-1 = 5/7; Lhx3 = 4/6) and NFPAs (Pit-1 = 3/4; Lhx3 = 4/4). Small groups of normal pituitary cells also expressed Pit-1, Lhx3, and CXCR4. However, we have to point out that Pit-1 expression could be underestimated by technical issues with the antibody used. The marker expression profiles of all individual tumors and normal pituitaries are detailed in Table 1.

This expression pattern (localization in restricted adenoma areas) suggests that these cells could represent a specific tumor cell subpopulation, possibly hPASCs. To address this hypothesis, we performed double immunofluorescence experiments to detect whether stem cell markers are expressed within GH-expressing cells, composing the mass of the tumor, or in less differentiated, hormone-negative cells, forming the putative hPASC compartment. In all GHomas analyzed, we did not observe co-expression of GH with Sox2, Oct4, or nestin (Fig. 2a). Similar results were observed in normal pituitaries, in which CD133- and Sox2-expressing cells were GH-negative (Fig. 2b).

Thus, a hormone-negative cell subpopulation expressing stem cell markers is present within hPAs with higher frequency than in normal pituitary.

Isolation of hPASCs from Postsurgical Specimens by Stem Cell-Permissive Medium Selection or CD133$^+$ Sorting

To demonstrate that the above-described hPA subpopulation fulfills the recognized features of hPASCs, we isolated and expanded these cells by selection in stem cell-permissive medium, an approach developed to isolate CSCs from solid tumors. We choose the medium formulation used to expand glioma stem cells (D-valine containing MEM/HAM'S F12 [1:1] supplemented with B27, bFGF, and EGF) [32], enriched with 1 % FBS and 10 ng/ml LIF, to maintain stemness in vitro [48].

Important limitations in studies using postsurgical hPA samples are the small number of cells available for in vitro experiments, the low proliferation rate, resulting in the impossibility to repeat experiments in cells from the same tumor, and in the limited number of assays feasible in each culture [49]. Thus, we analyzed a large number of hPAs ($n = 56$), performing different experiment sets in (unselected) adenoma subgroups (see Table S1). In this way, although not all experiments were performed on the entire hPA cohort, we achieved the characterization of both phenotypical and biological features of cells isolated from a significant number of adenomas, covering all the parameters required to define hPASCs [8, 50].

We obtained fibroblast-free, slow-growing cultures from 38 samples (10 GHomas, 1 mixed GH-PRL, 1 ACTHoma, and 26 NFPAs), with a success rate of 68 %. To obtain better adherence to the substrate, cells were grown on a coating of diluted Matrigel, a condition that allows stem cells to grow as monolayer, retaining their biological and phenotypical characteristics [37]. Before the experiments, the absence of contaminating fibroblasts was demonstrated in all cultures by ICF for procollagen I expression (data not shown). These results were confirmed by CD133 expression cell sorting on the whole hPA cell population using immunomagnetic selection ($n = 2$): CD133$^+$ subpopulation represented about 11 % of the total hPA cells in NFPA87 and 1 % in NFPA88 (Fig. S3A, B).

Pituitary cells grown in stem cell-permissive medium displayed the ability to survive and proliferate, as shown by Ki-67 labeling (Fig. S4A), for a prolonged time (up to 2 months), at odds with cells from the same tumors grown using standard medium (containing 10 % FBS) whose proliferation ability (if any) was limited, and in vitro survival lasted only few days, as reported [51, 52].

We quantified the ability of hPASCs to survive and duplicate in vitro by MTT assay, performing time course experiments in 11 hPA (two GHomas and nine NFPAs) cultures, weekly evaluating cell number up to 28 days. The analysis of all the cultures together showed a long-term proliferation that reached statistical significance starting from day 14 and persisting up to day 28 (Fig. 3a). However, the overall analysis underestimated the entity of the response due to the variable pattern of response among the cultures. In some cases (GH52, NFPA53, NFPA81, and NFPA82), the growth pattern was linear during the time of observation (7–28 days, with a maximal increase range of +199/+393 % vs. the number of cells at time 0). In others, cells were viable, but the increase in cell number was detectable only after 14 (NFPA54, NFPA66, and NFPA79) or 21 days (NFPA59). Finally, in other adenomas, cell proliferation

Table 1 Determination of stem cell/committed progenitor marker expression in human pituitary adenoma and normal pituitary sections by IHC analysis

Number	Code	Type	Sox2	Oct4	Nestin	CD133	Notch1	NANOG	CXCR4	Lhx3	Pit-1
Pituitary adenoma											
1	GH34	GH	+	+	+	+		−	+	−	+
2	GH36	GH	+	+	+	+	+	−	+	+	+
3	GH38	GH	+	+	+	+	−	−	+	+	+
4	GH46	GH	+	+	+	+	−	−	+	+	+
5	GH49	GH	+	+	+	+	+	+	+		−
6	GH52	GH	+	+	+	+	−	−	+	+	−
7	GH77	GH	+	+	+	+		−	+	−	+
8	NFPA43	NFPA	+	−	−	+	+	+	+	+	+
9	NFPA78	NFPA	+	+	+	+		−	+	+	+
10	NFPA79	NFPA	+	+	+	+		−	+	+	+
11	NFPA81	NFPA	+	+	+	+		+	+	+	−
12	NFPA82	NFPA	+		+			−	+		
Total		$n = 12$	12/12	10/11	11/12	11/11	3/6	3/12	12/12	8/10	8/11
% Positive			100	91	91	100	50	25	100	80	73
Normal pituitary											
1	A35		−	+	−	+	−	−	+	+	+
2	A23		+	+	−	+		−	+	−	+
3	IPO4		−	−	−				+	+	+
4	IPO5		+	+	+			−	+	−	+
Total		$n = 4$	2/4	3/4	1/4	2/2	0/1	0/3	4/4	2/4	4/4
% Positive			50	75	25	100	0	0	100	50	100

Blank boxes: not done

and/or survival, although still long-lasting for primary cultures of pituitary cells in vitro, declined after 14 (NFPA58) or 21 days (NFPA54 and NFPA78). Thus, although long-term proliferation and/or survival was identified in all cultures, differences within individual tumors are present. These results were confirmed in $CD133^+$-sorted cells (Fig. S3C).

The comparison of the proliferation rate of cells from the same hPAs, either selected in stem cell-permissive medium or grown in differentiation medium, showed that only stem-like cells were able to proliferate, while a significant cell number reduction occurred in differentiated cells starting from day 14 (Fig. 3b).

Sustained self-renewal is a main feature of tumor stem-like cells and is in vitro evaluated with the spherogenesis assay [25]. Isolated hPA cells selected in stem cell-permissive medium and grown without Matrigel formed spheroid aggregates in 14 of 16 cases (Fig. 3c), in one culture (NFPA81) forming loosely adherent colonies, a condition also observed in CSCs from malignant tumors [53], while only in GH63 was in vitro spherogenesis not detectable. SFE was calculated in a subset of samples (Fig. 3c) and ranged between 7 and 1.3 % (Fig. 3c). Pituisphere formation occurred after 7–10 days in culture, but proliferation within spheroids lasted for several weeks (Fig. S4A). Moreover, cell growth within pituispheres did not reduce self-renewal activity since cells harvested from disaggregation of 7-day-living spheroids generate secondary spheres after further 13 days in stem cell-permissive medium. In fact, the calculated SFE was slightly higher in secondary spheres in comparison with the primary SFE in the same adenoma (Fig. 3d). Secondary spheres were able to grow for two additional weeks (Fig. 3d). The detection of spherogenic activity in more than 90 % of the samples and its retention after several weeks in vitro further confirm that these cultures are enriched in hPASCs.

Phenotypic Characterization of hPASCs

To verify whether isolated cells represent the in vitro expansion of the rare stem cell marker-expressing subpopulations identified by IHC, we performed ICF analysis for the same markers in 20 hPA cultures. Sox2, Oct4, nestin, and CD133 were expressed in almost all of the cultures (Table 2) either grown as monolayer on Matrigel or as pituispheres (Fig. 4a, b). Moreover, with two exceptions (nestin in NFPA66 and CD133 in GH46), in which only few cells were labeled, almost all of the cells within each culture expressed the analyzed markers, suggesting that the in vitro cultures

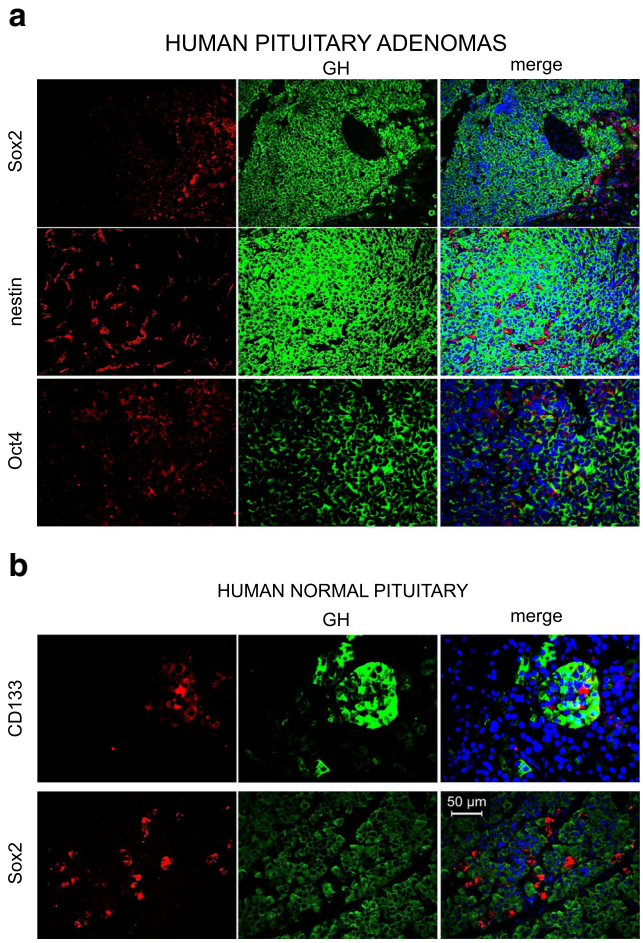

Fig. 2 Immunolocalization of putative stem cells in human GHoma (**a**) and normal pituitary (**b**) tissues by simultaneous assessment of stem cell marker and GH expression. **a** Double immunofluorescence for stem cell markers (*red*) and GH (*green*) in representative GHoma tissues shows the mutual exclusion of GH-expressing cells and either Sox2, nestin, and Oct4. Similar results were obtained in all the GHomas analyzed (see Table 1). **b** In normal pituitary, CD133 stem marker detection was confined to rare cells within GH-secreting cells (*yellow*) altogether with cells not expressing GH, while Sox2 is exclusively localized in GH-negative cells. Counterstain with DAPI is performed to highlight nuclei (*blue*). Original magnification: ×20 for panels in (**a**) and ×40 for panels in (**b**)

represent the expansion of the rare cells expressing stem cell markers identified in hPA sections; this was further confirmed by the analysis of $CD133^+$-sorted cells, which co-express Sox2, Oct4, and nestin in all of the cultured cells (Fig. S3D). Although identified in a low number of hPAs by IHC, Notch1 and NANOG expression was consistently detected in cell cultures (75 and 54 %, respectively). In the hPAs in which both tissue and cell culture were analyzed (see Tables 1 and 2), a concordant expression was detected in three cases (in GH52, both proteins were detected neither in tissues nor in cell cultures, and in NFPA43 and GH49, tissues and cells both express NANOG and/or Notch1). In others (GH34 and GH46), Notch1-positive cells were identified in culture, but not in histological sections, although, due to the small number of cells labeled, it is possible that IHC provided false negative data. Moreover, hPASCs express CXCR4 (in cells selected by growth in stem cell-permissive medium or $CD133^+$ sorting) and its ligand CXCL12 (GH49 and NFPA67), although the latter was tested only in two samples. Unexpectedly, also Lhx3 and Pit-1, markers of commitment to an endocrine phenotype of progenitor cells, were expressed by hPASCs (Fig. 4a, b).

Next, we tested the ability of hPASCs to differentiate into cells with an adult pituitary cell phenotype, evaluating the modulation of stem cell-like markers in cells selected in stem cell-permissive medium for 7 days and subsequently "differentiated" by shifting in FBS-containing medium for further 10 days. Differential marker expression between the two subpopulations was, indeed, observed (Fig. 5): CD133 and nestin expression was abolished in differentiated hPA cells, while the expression of Oct4 was significantly reduced. Conversely, the expression of CXCR4 was not modified and the number of $Lhx3^+$ cells was slightly increased. These results were confirmed by RT-qPCR in hPASC and differentiated cells isolated from the same adenoma: nestin, Oct4, and Sox2 messenger RNAs (mRNAs) were greatly more expressed in stem-like than in differentiated cells (+3.24-, +1.77-, and +2.21-fold, respectively); differences in NANOG and CD133 mRNA content were also evident, but at a lower extent (+1.48- and +1.44-fold vs. differentiated cells), while CXCR4 mRNA did not change in the two populations (Fig. S4B).

The main sign of pituitary cell differentiation is the induction of hormone expression. GHoma hPASC did not express GH, but after shifting in FBS-containing medium, GH immunoreactivity was induced in parallel with CD133 downregulation ($n = 2$; Fig. 5), supporting the differentiative potential of hPASCs. However, after 30 days in vitro (DIV), few GH-expressing cells appeared also in cultures kept in stem cell-permissive medium, although a much larger increase occurred switching the cells in FBS-containing medium (Fig. S5). Thus, a low level of spontaneous differentiation may occur in the hPASC subpopulation, although most of the cells retained the undifferentiated, stem-like phenotype.

Assessment of the Tumorigenicity of hPASCs

The designation of tumor stem-like cells requires the operative assessment of the ability to reproduce in vivo the tumor from which the cells have been isolated. Thus, we tested the tumorigenicity of hPASCs expanded in vitro for 10 days by inoculation in NOD-SCID mice. However, s.c. injection of cells into the flank of the animals in the presence of Matrigel (20×10^5 cells, $n = 2$) or pseudo-orthotopically in the striatum (50×10^5 cells, $n = 4$; Table S1) did not cause tumor

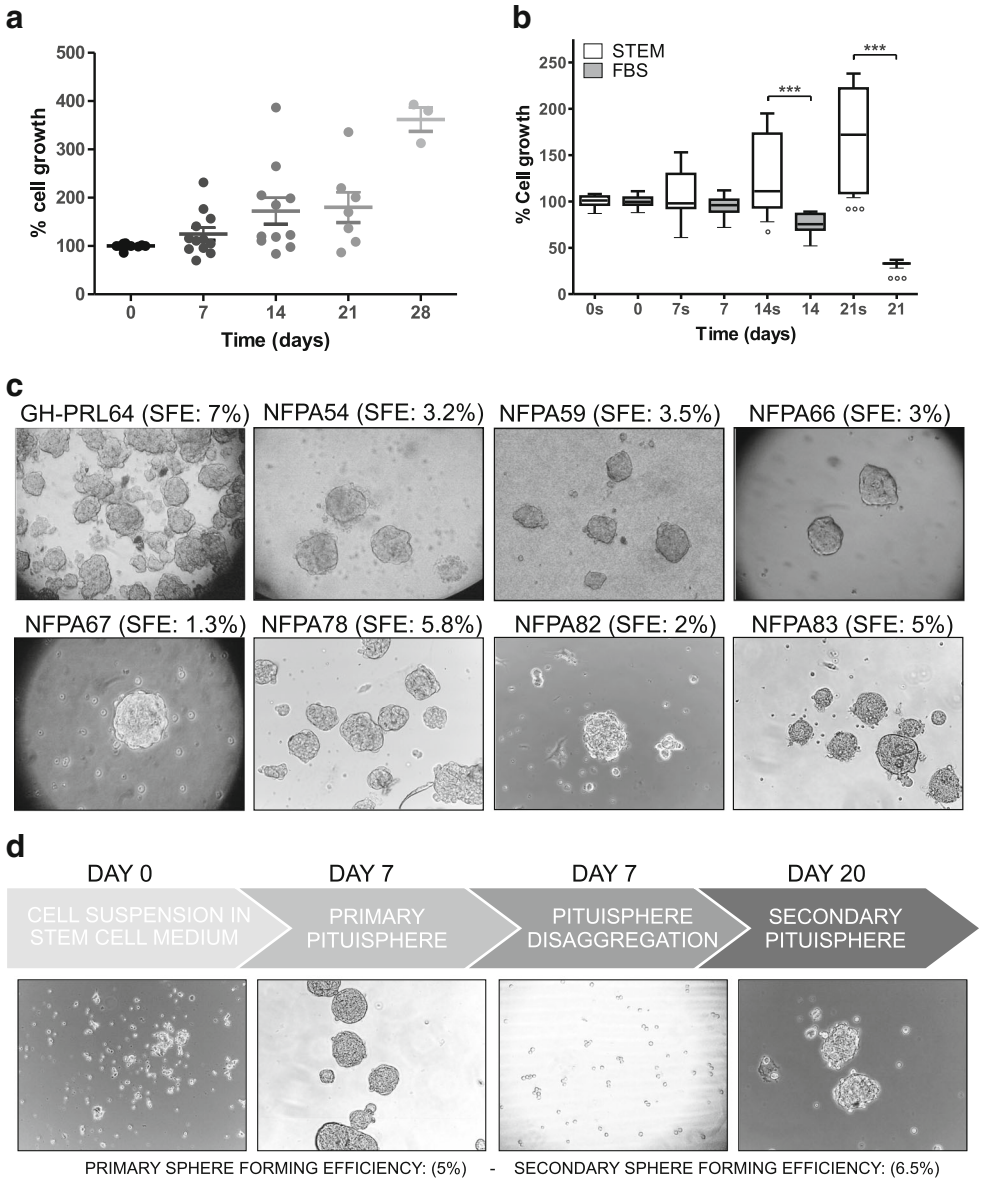

Fig. 3 In vitro proliferation activity and self-renewal of hPASCs grown in stem cell-permissive medium. **a** Growth curves of 11 hPASC cultures, evaluated every week for up to 4 weeks, demonstrate a sustained proliferation activity. Scatter dot plot graph depicts the mean ± SEM, expressed as the percentage of respective time 0. Each *point* represents one individual adenoma. The different number of samples tested at a given time point reflects the different availability of cells forming each hPA so that, for example, only a few cultures have enough cells to be tested for all the 4 weeks. (*t* test: 14 days $=p<0.05$; 21 days $=p<0.01$; 28 days $=p<0.001$ vs. day 0). **b** Comparison of the long-lasting proliferative activity of hPASCs and differentiated hPA cells. *Graph* depicts the cumulative growth time course of hPASC (*STEM*) and differentiated cells (*FBS*) derived from the same five hPAs (NFPA58, GH63, NFPA66, NFPA79, and NFPA85). A statistically significant increase in cell proliferation was observed in hPASCs, from day 14 to day 21 ($^{\circ}p<0.05$ and $^{\circ\circ\circ}p<0.001$ vs. day 0), while the corresponding differentiated cultures did not proliferate at any time point and the cell number significantly declined on day 21 ($^{\circ\circ\circ}p<0.001$); statistically significant differences between *STEM* and *FBS* cell growth was reached after 14 and 21 days in culture ($^{***}p<0.001$). *Box* and *whisker* plot depict the percentage of cell viability: *boxes* extend from the 25th to the 75th percentile, *lines* indicate the median, and *whiskers* extend to the minimum and maximum data points; statistical analysis was performed using ANOVA followed by post hoc Tukey's test. **c** Single hPASCs, maintained in the stem cell-permissive medium, generate homogenous pituispheres. Phase-contrast images of 7-day cultures are depicted from eight representative hPAs, out of 16 tested (magnification, ×20). Sphere-forming efficiency (*SFE*), representing the number of spheres formed every 100 cells plated, is indicated on top of the respective pictures. **d** Self-renewal assay: analysis of sphere formation from low density cell culture after dissociation of first-generation pituispheres and cell replating to generate second-generation spheres. In hPA cultures (NFPA67, NFPA78, and NFPA83, with representative images from the latter reported), 7-day primary spheres were dispersed, and secondary spheres were generated on day 20 in vitro (magnification, ×20). SFE was slightly increased in the secondary sphere assay

development within 8 months. Thus, we hypothesized that, in agreement with the observation that most of the adenomas are identified as incidentalomas [54], the low proliferation rate of hPA cells might determine a tumor development too slow to

Table 2 Determination of stem cell/committed progenitor marker expression in human primary pituitary adenoma stem-like cultures by ICF analysis

Number	Adenoma code	Type	Sox2	Oct4	Nestin	CD133	Notch1	NANOG	CXCR4	Lhx3	Pit-1
1	GH33	GH					+	+	+		
2	GH34	GH					+	+	+		
3	GH46	GH	+			RARE	+	RARE	+		
4	GH49	GH	+		+	+	+	+	+		
5	GH52	GH	−	+	+	+	−	−		−	+
6	GH63	GH	+	+	+	+			+	+	+
7	NFPA43	NFPA	+		+	+	+				
8	NFPA51	NFPA	+	+	+	+	+	−		+	+
9	NFPA57	NFPA	+	+	+	+	−	−	+	+	+
10	NFPA59	NFPA	+	+	+				+	+	+
11	NFPA66	NFPA		+	RARE	+			+	+	+
12	NFPA67	NFPA		+	+	+			+	+	+
13	NFPA73	NFPA				+			+	+	
14	NFPA78	NFPA	+	+					+	+	+
15	NFPA81	NFPA	+	+	+			+	+		
16	NFPA82	NFPA		+	+						
17	NFPA83	NFPA		+	+				+		
18	NFPA84	NFPA	+	+	+			+	+	+	
19	NFPA85	NFPA	+	+	+			−	+		
20	NFPA86	NFPA	+	+	+			+	+		
21	NFPA87	NFPA	+	+	+	+					
Total		$n = 21$	13/14	15/15	14/16	10/11	6/8	6/11	16/16	9/10	8/8
% Positive			93	100	87	91	75	54	100	90	100

Blank boxes: not done. When only a few cells resulted positive for a given marker (labeled in the table as RARE), the culture was considered negative

be detected in mice. Since the in vivo assessment of tumorigenesis is an absolute requirement to define tumor stem-like cells, we tested this parameter in zebrafish embryos, which allows the detection of the in vivo biological behavior of tumor cells within a shorter time, more compatible with the hPA cell characteristics [55]. hPASCs ($n = 5$, although only three were statistically evaluable) were xenografted in zebrafish embryos (48 h post-fertilization, 20 embryos/hPASC culture) using the Tg(*fli1*:EGFP)^{y1} zebrafish line that expresses EGFP in the vascular endothelium [39]. In this model, in which only a small number of cells (0.5×10^3/embryo) is required and all the events occur within 2–3 days [40], the assessment of tumorigenesis is indirectly derived by the analysis of the invasive and neoangiogenic potential of the injected cells. Grafted red fluorescence-stained hPASCs showed a strong invasive behavior: starting 24 hpi, pituitary cells migrated out from the tumor mass at the injection site and invaded different parts of the embryo, in particular the area of the posterior caudal vein plexus (Fig. 6a–c). Moreover, while control embryos did not display alterations of vascular network, grafted embryos showed vessels that sprout from the subintestinal vein plexus toward the tumor cells after 24 hpi (Fig. 6d–f) and 48 hpi (Fig. 6g–i), demonstrating that hPASCs stimulate in vivo neoangiogenesis. Moreover, IHC analysis on grafted embryos confirmed that tumorigenic hPASCs express CD133 (Fig. 6j–m).

Expression of Somatostatin and Dopamine Receptors in hPASCs and their Role in Cell Proliferation and Survival

The main pharmacological approach for hPAs involves somatostatin and/or dopamine agonists, which, besides the inhibition of hormone hypersecretion, might control cell proliferation by the activation of specific phosphotyrosine phosphatases [56–58], although this effect is inconstantly observed in vivo. The currently approved drugs, octreotide/lanreotide and cabergoline, are mainly effective on SSTR2 and SSTR5 (whose expression in hPA is depicted in Fig. 7a), and D2R, respectively. Thus, we verified the expression of these receptors in hPASCs. By IHF in hPA sections, we show that nestin-expressing cells are also SSTR2$^+$ (Fig. 7b). Similarly, in vitro hPASCs grown in monolayer (data not shown) or as pituispheres (Fig. 7c) co-expressed SSTR2, SSTR5 or D2R,

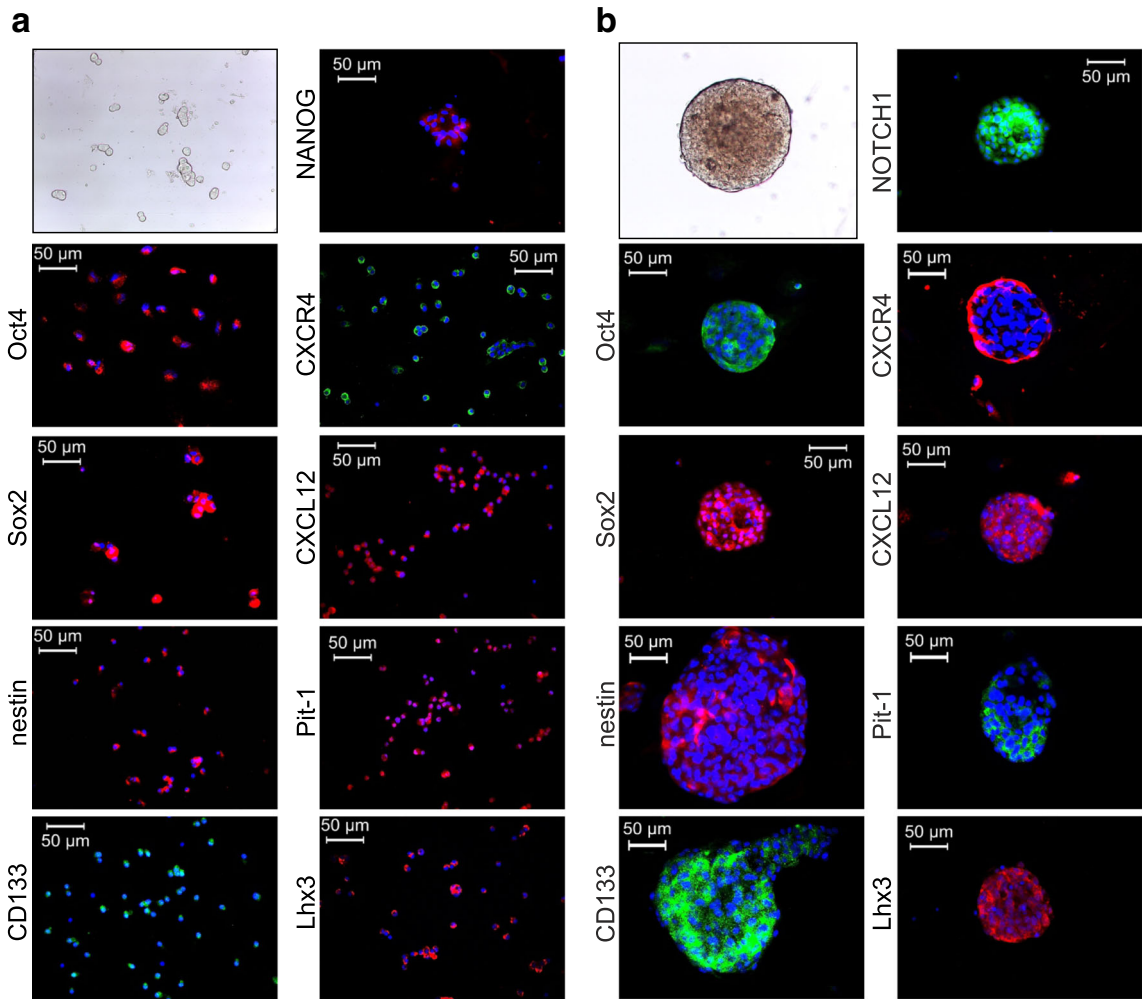

Fig. 4 Stem cell marker expression is enriched and maintained in hPASCs after selection and expansion in vitro. Phase-contrast representative micrographs of hPA cells cultured in stem cell-permissive medium: *upper left image* depicts the morphological appearance of hPASCs grown as monolayer (**a**) or as pituispheres (**b**) (magnification, ×40). hPASC culture immunocytofluorescence from 21 adenomas (Table 2) shows the expression of stem cell markers and pituitary stem/progenitor transcription factors in almost all cells within monolayer (**a**) and pituispheres (**b**), suggesting the selection and in vitro expansion of stem-like cells from the original tumors. Negative controls are reported in Fig. S1C

and Oct4 or nestin. Thus, we evaluated the effects of the activation of these receptors in seven hPASC cultures using the somatostatin/dopamine chimera BIM-23A760 [59]. In all the cultures analyzed, BIM-23A760 (1 nM) inhibited cell survival (range = −14 to −30 %, of vehicle-treated controls), which was statistically significant in six of seven cultures (Fig. 7d). Interestingly, analyzing the effects of BIM23A760 in cells kept growing in vitro for different times (7, 10, 13, 27, and 30 DIV), we observed that cell responsivity remained unchanged even after prolonged time in culture (Fig. 7d). In two cultures, in which the yielded cell number allowed concentration–response experiments (Fig. 7e), we show, in one case, that BIM-23A760 effect was concentration-dependent (0.1–100 nM), with a maximal effect of about −43 %, while in the other, no concentration dependency was observed, although cell survival was inhibited at low concentrations (10 pM).

Discussion

While proposed since several years, the presence of hPASCs (stem-like cells in hPAs) has been reported only in a few studies, without reaching concordant results [26–28, 60]. Interestingly, IHF data from hPA sections in the published studies show more homogeneous results with the data here described than the evidence provided in vitro. In particular, in agreement with previous reports [27, 60], our study shows that cells expressing markers of stemness (CD133, nestin, Oct4, Sox2, Notch1, and NANOG) are present in discrete areas of most GHomas and NFPAs. Moreover, in line with our study, Xu and colleagues reported in two hPAs [26] and Chen and colleagues in 20 [27] that pituispheres are enriched in CD133- and nestin-expressing cells. The immunohistochemical co-localization of these markers in hPA has also

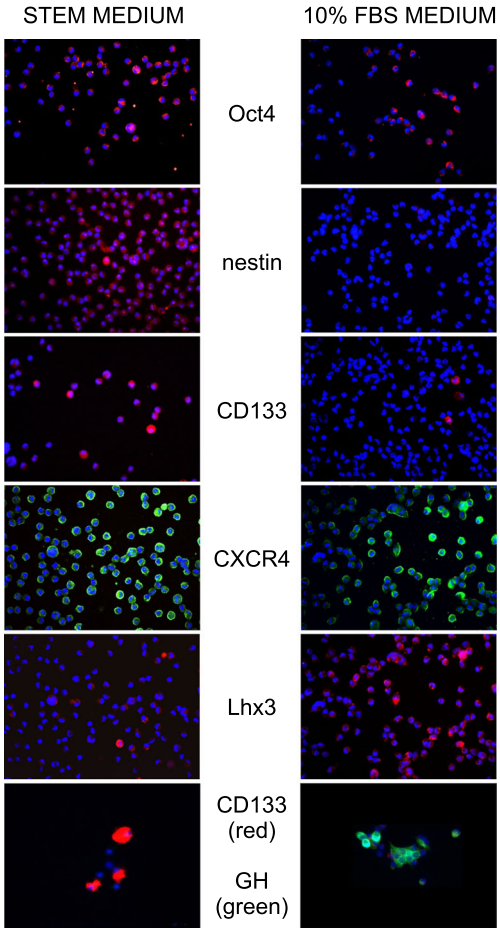

Fig. 5 hPASCs are able to differentiate in stem cell marker^{low}/GH^{+} cells. Downregulation of specific stem cell markers (Oct4, nestin, and CD133) in differentiated hPA cells (grown in the presence of 10 % FBS, *right panels*) compared to their hPASC counterparts (*left panels*), assessed by immunocytofluorescence in GH49, GH52, and GH63. CXCR4 expression was not modified in differentiated cells, while Lhx3 slightly increased. The differentiation potential of hPASCs is further corroborated by the induction of GH expression. Nuclear counterstain was performed with DAPI. Negative controls are reported in Fig. S1C. Original magnification, ×40

been described [60]. However, several other stem cell markers label these cells, as variably documented by our (Oct4, Sox2, Notch1, and NANOG) and previous studies (Oct4, Musashi, Notch4, and Jag2 [26]; Oct4, NANOG, Klf4, and Sox2 [28]; Tuc, Dcx, and βIII-tubulin [27]), supporting the notion that hPAs contain undifferentiated stem-like cell subsets, likely sustaining tumor growth. Moreover, we report that, similarly to rat and/or mouse normal pituitary or in genetically induced adenomas [30], stem cell marker-expressing cells within hPAs do not co-localize with hormone-expressing cells, an evidence highly suggestive that they represent stem/progenitor tumor cells.

We demonstrate that this minor hPA subpopulation can be expanded in vitro for several passages. In fact, growing cells dispersed from 38 adenomas in stem cell-permissive medium, we obtained a homogeneous cell culture enriched in stem-like cells, displaying cell division ability, albeit at a low rate, for several weeks, self-renewal potential (as for the ability to grow as pituispheres), and stem marker expression (CD133, nestin, Sox2, Oct4, NANOG, and Notch1) in almost all cells. These results are in line with a recent study, using transgenic Rb$^{+/-}$ mice that naturally develop pituitary adenomas [61], evidencing that a subpopulation isolated from these tumors as Sca1^{+}, also expressing CD133, nestin, and Sox2 [30], displayed similar hPASC features as here described in cells from human samples. Importantly, although several pituitary stem cell markers are retained in hPASCs (i.e., Notch1 and Sox2, whose expression controls pituitary development, but are less relevant for the homeostasis in adult pituitary) [15], their phenotype does not completely recapitulate that of normal pituitary stem cells. In fact, we identified, besides bona fine pituitary stem cell markers, different CSC markers previously identified in several malignant tumor histotypes (CD133) or pituitary markers identified in differentiation-committed precursors (Lhx3 and Pit1). Although unexpected, these results are compatible with the notion that, with the exception of adamantinomatous craniopharyngiomas [16], tumor stem-like cells do not necessarily derive from the stem cells of the relative adult tissue and that often the aberrant expression of specific markers is detected. However, the stem-like properties of these cells were demonstrated by the observation that differentiation of hPASCs abolished the expression of stem-like markers (CD133, nestin, and Oct4) and induced (in GHoma-derived cells) GH expression. These data clearly suggest that hPASCs can give origin to the bulk of cells composing pituitary adenoma mass, losing stemness and acquiring commitment to hormone-secreting ability. Moreover, their persistence within the adenomas grants the tumors with a reservoir of hormone-secreting tumor cells.

Although expected differences were observed among individual tumors, we demonstrate that only hPASCs retain sustained proliferation activity in vitro and survived for several weeks. In contrast, differentiated hPA cells, representing the population that composes the adenoma mass, show low/null proliferation potential. Nevertheless, despite that we tried to enrich the culture medium with LIF, a cytokine reported to sustain cell stemness [48], our cultures were not unlimited: cell growth lasted, at most, for 2 months, before the inevitable decline, concomitant with the spontaneous differentiation into GH-producing cells. This problem, not observed when culturing stem cell from malignant tumors (i.e., glioblastoma) [32, 37], could be ascribed either to the intrinsic features of stem-like cells from hPA or to suboptimal culturing conditions. Further studies are required to address this issue.

The hPASC division rate, although long-lasting, was rather slow, with an average population doubling time of about 14 days. This evidence was at odds with a previous study reporting a rapid doubling time of putative hPASCs [28]. However, in that study, isolated cells displayed no pituitary

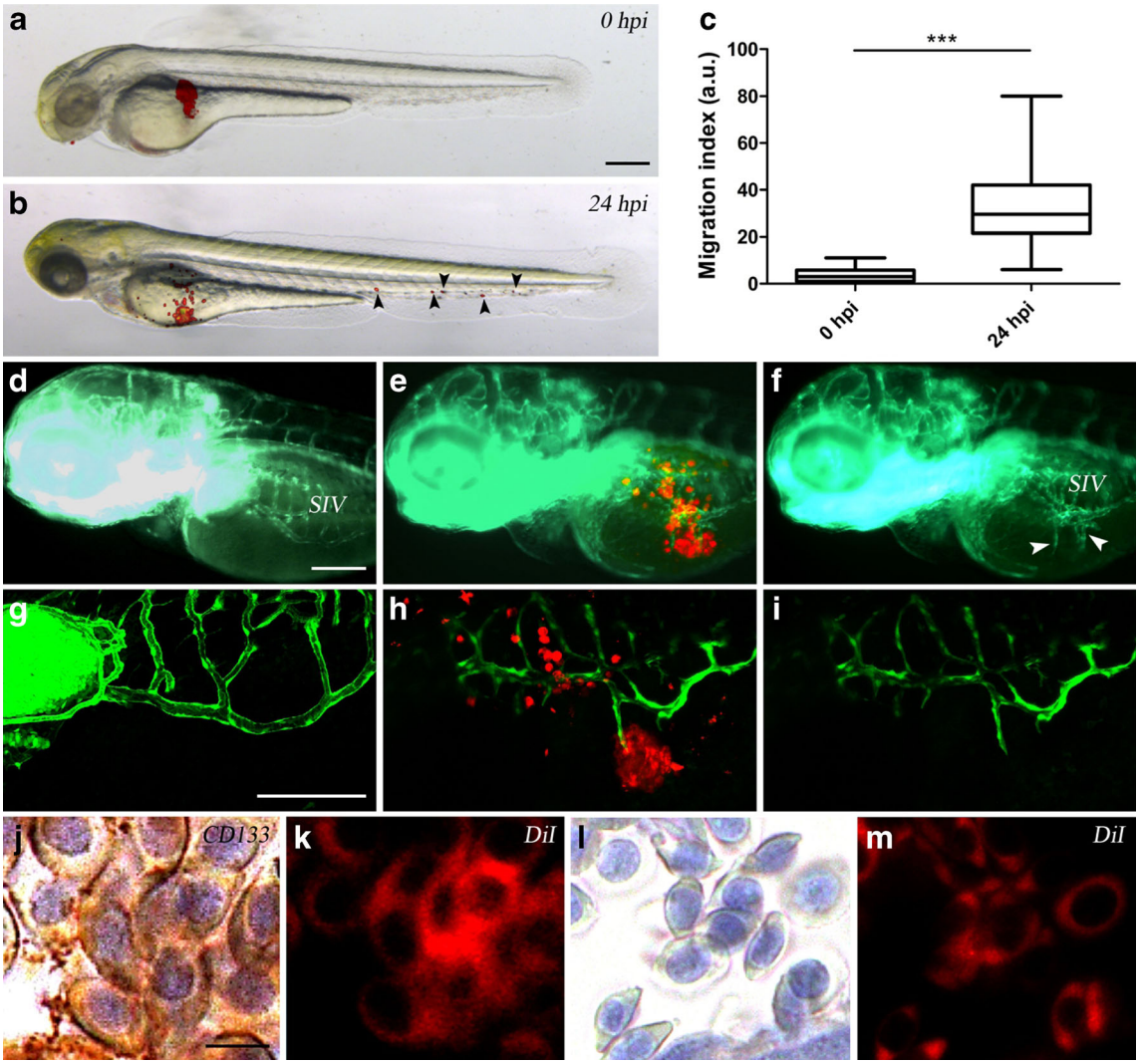

Fig. 6 hPASCs are tumorigenic in zebrafish embryos, showing invasiveness and angiogenic potential. **a**, **b** Overlay of representative fluorescent and bright-field images of grafted embryos at 0 (**a**) and 24 h after injection (hpi) (**b**) shows the spread of red-stained hPASCs throughout the embryo body. *Black arrowheads* indicate migrating cells in the area of the posterior caudal vein plexus. *Scale bar*, 300 μm. **c** Box plot showing the quantification of hPASC invasiveness. The migration index was calculated as the mean number of red clusters in grafted embryos at 0 (**a**) and 24 hpi (**b**). (*t* test: ***$p < 0.0001$). **d**–**i** hPASCs stimulate angiogenesis in zebrafish embryos. Epifluorescence images at 24 hpi (**d**–**f**) and confocal images at 48 hpi (**g**–**i**) compare PBS-injected control embryos (**d**, **g**) and hPASC (*red*) xenografted embryos (**e**, **f**, **h**, **i**) in which endothelial structures (*green*) sprouting from the subintestinal vein reach the tumor mass. In (**f**) and (**i**), the red channel was omitted to highlight the newly formed endothelial sprouts (*white arrowheads*). All images are oriented so that rostral is to the *left* and dorsal is at the *top*. *Scale bar*, 100 μm. **j**–**m** Tumorigenic hPASCs express CD133. IHC analysis of sections of grafted embryos shows that red-stained hPASCs (**k**) are positive for CD133 (**j**). In (**l**) and (**m**), a representative field from the grafted embryo used as a negative control (no primary antibody) is reported. *Scale bar*, 10 μm

markers, fibroblast-like morphology, mesenchymal stem cell phenotype, and the ability to differentiate in osteoblasts, adipocytes, and chondrocytes [28], suggesting that this subpopulation might derive from cells unrelated to pituitary adenoma parenchyma, possibly pericytes or stromal precursors.

Besides the in vitro characterization, the only parameter that operationally defines tumor stem-like cells is the ability to reproduce in vivo the tumor from which they derive [50]. To date, only two studies reported tumor development in mice after injection of putative hPASCs [26, 27]; we were not able to induce pituitary tumors by injecting hPASCs in the brain or s.c. in NOD/SCID mice, and a recent paper confirmed this negative findings injecting both GHoma and NFPA hPASCs either s.c. or under the kidney capsule [29]. In these conditions, cells apparently survived a few months, but no clear proliferation was observed. While we do not have an explanation on why in the previous studies adenoma cells were able to grow in vivo, we have to point out that, in those studies, cells from only two [26] or one [27] hPA were reported to grow in mice. This low number may reflect particular conditions occurred in hPASC selection, providing cells with unusual tumorigenic potential. On the other hand, we

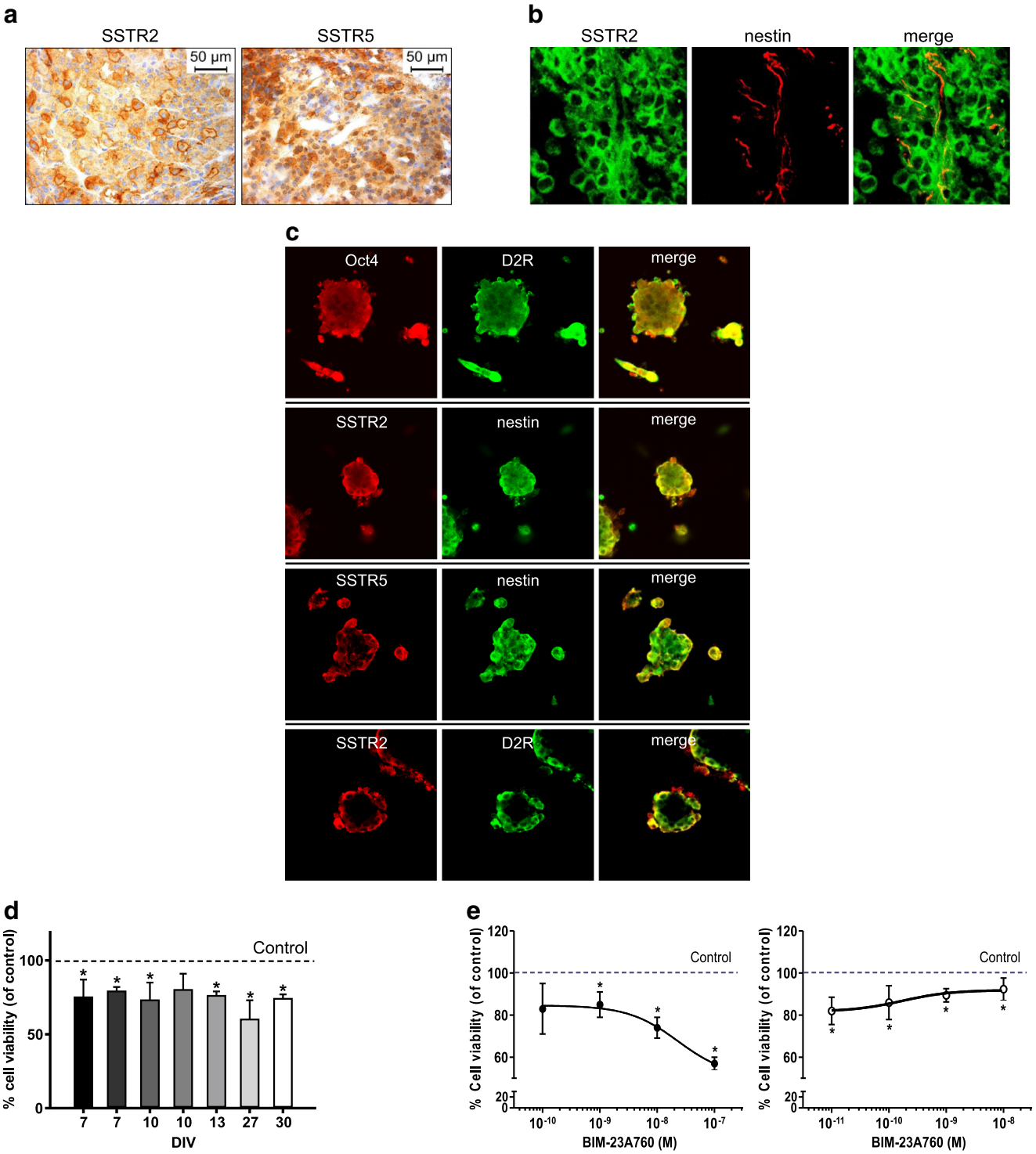

Fig. 7 hPASCs express functional somatostatin and dopamine receptors, which mediate antiproliferative signals. **a** Representative pictures from IHC analysis showing the membrane localization of SSTR2 and SSTR5 within adenoma tissues. Magnification, ×40. **b** Representative pictures from confocal analysis on adenoma tissues showing co-localization of SSTR2 (*green*) with nestin (*red*). Magnification, ×120. **c** Confocal images from double immunofluorescence for SSTR2, SSTR5, and D2R in pituispheres co-labeled with Oct4 and nestin antibodies. Pituitary adenoma cells selected and in vitro expanded in stem cell-permissive medium form pituispheres that co-express somatostatin and dopamine receptors with stem cell markers (Oct4 and nestin). **d** Antiproliferative effects of BIM-23A760, a chimeric molecule agonist for somatostatin and dopamine receptors, in seven hPASC cultures, grown in vitro for different times (7, 10, 13, 27, and 30 days in vitro, *DIV*; *t* test: $*p < 0.05$). **e** Dose–response curves from two hPASC cultures (ADE83, *left*; ADE86, *right*) of BIM-23A760 (10^{-11}–10^{-7} M). The dose-dependent effects of BIM-23A760 on cell survival was evaluated by MTT assays, run in triplicate for each point, and the percentage of inhibition was calculated against vehicle-treated control. Data are expressed as the mean ± SEM after 24 h of treatment (Dunnett' test: $*p < 0.05$)

hypothesize that, pituitary tumorigenesis being a rather slow process, it could be easily missed when analyzed in immunocompromised mice, being not compatible with the animal life span. Thus, we choose zebrafish as an alternative in vivo model. hPASCs grafted in zebrafish embryos, if endowed with tumorigenic potential in vivo, should display invasive behavior, colonizing significant areas of the embryo, including the tail, and will promote neoangiogenesis, as shown by the neovessels directed toward the mass of grafted cells [62]. We indeed demonstrate that hPASCs have invasive and proangiogenic activity in vivo, strongly supporting their tumor stem-like cell nature. Unfortunately, the hPA cell number available for these experiments did not allow the comparison of the in vivo behavior of hPASCs and differentiated adenoma cells. Thus, the occurrence of a different biological activity in vivo of hPA cell subpopulations will require further analysis.

According to the CSC theory, stem-like cells represent the main drug target required to eradicate tumors, and recently, several molecules were identified as inhibitors of CSC viability (salinomycin, metformin, sorafenib, etc.) [36, 63, 64]. We demonstrate that the established hPA drug targets (i.e., SSTR2/5 and D2R) commonly used to control pituitary hormone hypersecretion are also expressed in hPASCs. This observation suggests that these cells are likely not derived from undifferentiated normal pituitary stem cells but from cells that already entered the differentiation process (as also shown by Pit-1 expression in these cells). Moreover, since SSTR2/5 and D2R activation by the dopastatin BIM-23A760 [59] induces antiproliferative effects, it is possible that the inconstant antitumoral activity observed in hPA patients treated with agonists for these receptors is dependent of the relative fraction of hPASCs present in each tumor.

To sum up, in agreement with the hierarchical model of tumorigenesis, we propose that stem-like cells are present in both GHomas and NFPAs. These cells drive tumor development and, due to the long-lasting proliferation activity, act as a reservoir to originate the differentiated hormone-secreting cells composing the adenoma mass, which, after losing the stemness potential, enter in a senescence program, the mechanism that determines the benign nature of hPAs [65]. We provide evidence that cells composing this subpopulation fulfill all the defining features of tumor stem-like cells, namely, expression of stem markers, long-term proliferation and survival in vitro, self-renewal, and tumorigenicity in vivo, at least as far as the ability to promote neoangiogenesis and invasiveness. Although expected differences among tumors were observed, these data were reproduced in cells isolated from a high number of hPAs, suggesting that the presence of hPASCs represents a common feature in these tumors. Thus, we propose that the tumor stem-like cell theory could be extended to some benign and non-aggressive tumors. We demonstrate that hPASCs retain the expression of somatostatin and dopamine receptors and, therefore, are a good target for the currently available pharmacological approaches. Finally, the isolation of tumorigenic subpopulations within hPAs may open new avenues for the identification of novel drug targets to improve the pharmacological outcome of the currently available treatments.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

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Zebrafish as an innovative model for neuroendocrine tumors

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Abstract

Tumor models have a relevant role in furthering our understanding of the biology of malignant disease and in preclinical cancer research. Only few models are available for neuroendocrine tumors (NETs), probably due to the rarity and heterogeneity of this group of neoplasms. This review provides insights into the current state-of-the-art of zebrafish as a model in cancer research, focusing on potential applications in NETs. Zebrafish has a complex circulatory system similar to that of mammals. A novel angiogenesis assay based on the injection of human NET cell lines (TT and DMS79 cells) into the subperidermal space of the zebrafish embryos has been developed. Proangiogenic factors locally released by the tumor graft affect the normal developmental pattern of the subintestinal vessels by stimulating the migration and growth of sprouting vessels toward the implant. In addition, a description of the striking homology between zebrafish and humans of molecular targets involved in tumor angiogenesis (somatostatin receptors, dopamine receptors, mammalian target of rapamycin), and currently used as targeted therapy of NETs, is reported.

Key Words
- zebrafish
- neuroendocrine tumors
- tumor xenografts
- angiogenesis
- somatostatin receptors

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Introduction

In the past decades zebrafish (*Danio rerio*) has emerged as a powerful vertebrate model system to study vertebrate developmental mechanisms. Indeed, zebrafish has a high fecundity (a female can lay up to 100–200 eggs/week), the embryos develop outside the body and are transparent, facilitating the observation of morphogenetic movements and organogenesis in real time (Pistocchi *et al.* 2008, Bellipanni *et al.* 2010, Quaife *et al.* 2012).

More recently, tlhe zebrafish has become an attractive model for the research on several human diseases including cancer (Liu & Leach 2011, Malafoglia *et al.* 2013). Although there are evident structural and physiological differences between zebrafish and humans, the zebrafish provides several advantages when compared with other vertebrate model systems (Lieschke & Currie 2007, Fieramonti *et al.* 2012, Konantz *et al.* 2012, Santoriello & Zon 2012).

This review provides insights into the current state-of-the-art of zebrafish as a model in cancer research, focusing on potential applications in neuroendocrine tumors (NETs).

Zebrafish as a cancer model

Although fish do not have certain organs found in mammals (breast, prostate, and lung), zebrafish spontaneously develops almost any type of tumor (Nicoli *et al.*

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2007). In addition, there is a high degree of histological similarity between tumors developed in zebrafish and those in human and many aspects of carcinogenesis are conserved in fish as compared with humans (Amatruda *et al*. 2002). In fact, despite zebrafish diverged from mammals during evolution about 450 million years ago, the developmental and genetic programs between these organisms are largely conserved (Liu *et al*. 2002).

Several strategies have been used to generate cancer models and to identify cancer-related genes in zebrafish: treatment with chemical carcinogens, forward genetic screening, reverse genetic approaches, transgenic models, and xenotransplantation of mammalian cancer cells (Tobia *et al*. 2011, Shive 2013).

Like their human and murine counterparts, zebrafish are susceptible to develop a significant number and wide variety of neoplasms after the exposure to chemical carcinogens (Feitsma & Cuppen 2008). Treating fish with carcinogens is very easy to set-up because the water-soluble carcinogens can be added to the fish water and embryos, larvae, and adult animals can be exposed for longer time periods (Feitsma & Cuppen 2008). Although the routes of exposure to carcinogens may differ between fish and mammalians, the liver is the primary target for many carcinogens in both fish and rodents (Shive 2013).

Zebrafish is one of the best vertebrate model currently used for forward genetic screening in order to identify cancer susceptibility genes. Mutations are induced in the zebrafish genome by carcinogens, irradiation, or viral/transposon-based vectors (insertional mutagenesis). The progeny of mutagenized fish are screened for cancer phenotypes. Mutated genes are identified through genetic mapping, sequencing analysis, and phenotype validation (Liu & Leach 2011). A forward chemical screen using zebrafish embryos may provide an alternative approach to identify cancer-susceptibility genes during embryogenesis, considering that several cellular pathways involved in cancer play also a role in embryonic development (Liu & Leach 2011). In addition, zebrafish forward-genetic screens are simplified by the optical transparency of embryos and larvae, a feature that facilitates the screening for cancer phenotype without sophisticated equipments (Lieschke & Currie 2007).

Reverse genetics is another strategy consisting in the modification of a gene of interest, or its expression, to analyze the phenotypic effects. The genetic versatility of zebrafish system and the recent technological innovations in genetics have transformed zebrafish into a sophisticated reverse genetic system, offering the possibility to increase our knowledge in the field of cancer. Several approaches are used to evaluate the effect of specific gene mutations on cancer development.

Targeting Induced Local Lesions in Genomes (TILLING) is a technique, in which genomic DNA from a large library of ethylnitrosourea-mutagenized zebrafish are screened for specific mutations in genes of interest. Screening is performed by PCR amplification of specific exons from each mutagenized zebrafish followed by mutation detection through direct resequencing of PCR fragments or alternatively, by CEL1 endonuclease-mediated mutation discovery (Liu & Leach 2011, Shive 2013). Once a mutant of interest is identified, individuals isolated from the library and mutant lines are established (Moens *et al*. 2008). The rapid advancements in next generation sequencing platforms, able to increase the speed and to reduce the cost of DNA sequencing, have recently increased the efficiency of mutation discovery for TILLING from mutant libraries (Santoriello & Zon 2012). However, this technique is laborious and time-consuming for a regular laboratory. Therefore, the Sanger Institute has set up a project called 'Zebrafish Mutation Project (ZMP)' with the aims to create a knockout allele in every protein coding gene in the zebrafish genome, using a combination of whole-exome enrichment and Illumina next generation sequencing. Mutations for 11 892 genes (about 45% of all zebrafish genes) have been identified by this project so far (http://www.sanger.ac.uk/Projects/D_rerio/zmp/).

Several emerging technologies are currently able to create targeted knockout mutants in zebrafish, such as zinc-finger nuclease-targeted mutagenesis, transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)–Cas (CRISPR-associated proteins) system. Zinc finger endonucleases consist of a DNA-binding zinc finger protein fused to a nonspecific cleavage domain of the FokI endonuclease. They can induce double-strand breaks that are generated by FokI endonuclease upon binding to specific DNA sequences recognized by the zinc-finger motifs. These damages are imprecisely repaired by nonhomologous end joining a DNA repair pathway frequently causing small insertions or deletions at the break site. Therefore, engineered zinc-finger nucleases can be designed to deliver frameshift mutations at specific sites in the genome of the zebrafish (Liu & Leach 2011, Santoriello & Zon 2012, Shive 2013).

TALENs are important new tools for genome engineering. TALENs are chimeric nucleases generated by a transcription activator-like (TAL) effector DNA-binding domain, constructed to bind any desired DNA sequence fused to a DNA cleavage domain. This system enables

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targeted gene disruption in a wide variety of model organisms, is easier to design and assemble compared with zinc-finger nucleases (Santoriello & Zon 2012, Shive 2013). Recent works have reported that TALENs can induce mutations in endogenous zebrafish genes, showing a high efficiency in inducing locus-specific DNA breaks in somatic and germline tissues (at some loci this efficacy approaches 100%) (Bedell *et al*. 2012, Ma *et al*. 2013).

Another innovative system for targeted genome engineering derived from the CRISPR–Cas defense. CRISPR–Cas constitutes an adaptive immune system used by bacteria and archaea against invading foreign nucleic acids derived from bacteriophages or exogenous plasmids. This defense system can incorporate specific short sequences of foreign nucleic acids into a region of the host genome that is distinguished by CRISPR. When these sequences are transcribed and processed into small RNAs, they guide a multifunctional protein complex (Cas proteins) to recognize and destroy incoming foreign genetic elements in a sequence-specific manner (Bhaya *et al*. 2011). Bacterial type II CRISPR systems can be engineered to direct targeted double-stranded DNA breaks *in vitro* to specific sequences by using a single 'guide RNA' with complementarity to the DNA target site and a Cas9 nuclease in mammalian cells (Cong *et al*. 2013). This system also works efficiently *in vivo* for inducing targeted alterations into endogenous genes in zebrafish with a somatic targeting efficiency similar to those obtained using zinc-finger nucleases and TALEN (Hwang *et al*. 2013).

A morpholino technology is routinely used in zebrafish to perform a transient gene knockdown. Morpholinos are synthetic antisense oligonucleotides which replace the ribose rings of RNA with morpholine rings. This modification enables morpholinos to be resistant to nuclease digestion and to increase binding activity to their complementary RNA sequences. Therefore, using a specific antisense morpholino, it is possible to target a selected transcript and to dramatically reduce the levels of the corresponding functional protein (Bill *et al*. 2009). Nevertheless, once injected into the embryos, the effect of morpholinos lasts only few days and thus this technique is not suitable for the study of loss-of-function consequences beyond the larval period.

Transgenic animals have provided the tools for exploring the effects of oncogene overexpression or tumor–suppressor gene inactivation (via dominant-negative strategies) on tumor phenotype. Several transgenic zebrafish models of cancer have been developed by microinjection of specific mammalian oncogenes in early-stage zebrafish embryos using transposon-mediated systems, supporting that most of tumorigenic mechanisms are conserved from zebrafish to human (Lieschke & Currie 2007). Injection of foreign DNA into fertilized eggs results in germline transgene integration with a high efficiency. Interestingly, tissue-specific and/or inducible transgenic methods have been successfully used in zebrafish to induce a specific type of cancer and to regulate the timing of tumor initiation. Indeed, different tissue-specific promoters and systems able to regulate gene expression with a high degree of temporal and spatial precision have been adopted in zebrafish, such as Tol2 transposon and the mifepristone-inducible LexPR, GAL4-UAS, and Cre-LoxP systems (Santoriello & Zon 2012, Mimeault & Batra 2013). In this frame, transgenic animals have led to experiments probing overexpression of WT, constitutively active, or dominant negative versions of a gene of interest (Santoriello & Zon 2012).

Xenotransplantation of human or mouse cancer cells into zebrafish represents another interesting tool mainly devoted to study *in vivo* tumor angiogenesis, invasiveness, and metastatic dissemination (Nicoli *et al*. 2007).

Although murine xenotransplant model remains the gold standard for studies in the field of human cancer research and drug development, there are several limitations associated with this model: long duration of time required to have a visible tumor implant and to perform experiments (from several weeks to months); requirement of a skilled technician for the complexity of several procedures; immunosuppressed mice are required to avoid transplant rejection, these animals are more susceptible to infection and drug toxicity than normal mice and need specific housing and care; its laborious and time-consuming process makes this model very expensive; large number of cells (about 1 million) are required to generate a tumor, making it less suitable as a xenotransplant model using primary tumor cells; high difficulties to generate mouse xenotransplant models able to metastasize (Haldi *et al*. 2006, Konantz *et al*. 2012).

The zebrafish xenotransplantation model cannot replace the use of mammalian model systems; however, it can overcome some of these drawbacks previously reported, providing a solid and complementary approach to mouse model. Experimental models have been established in zebrafish embryos, juveniles, and adults, each one with its own advantages and limits (Lieschke & Currie 2007).

Zebrafish is an amenable model system for vascular biology studies. Indeed, vessel/emathopoietic genetic program is largely conserved during evolution. Furthermore, zebrafish embryos are so small that they can receive enough oxygen by passive diffusion to survive and

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develop, reasonably normally, for several days in the complete absence of blood circulation (Isogai *et al*. 2001).

In embryos, vessels formation can occur by two different processes, vasculogenesis and angiogenesis. During vasculogenesis, endothelial cells differentiate from mesodermal precursors and proliferate *in situ* within a previously avascular tissue to form a primitive tubular network. Angiogenic remodeling refers to the process by which this initial network is modified to form the mature vasculature. In particular, angiogenesis occurs in the formation of the intersomitic vessels (ISVs) of the trunk, that sprout from the dorsal aorta, as well as of subintestinal vessels (SIV) originating from the duct of Cuvier area (Fig. 1A and B; Isogai *et al*. 2001). A further vessel present in this region is the common cardinal vein (CCV) that fans out across the yolk on either side (Fig. 1A and B; Isogai *et al*. 2001). Moreover, zebrafish possess a lymphatic system that shares many of the morphological, molecular, and functional characteristics found in other vertebrates (Yaniv *et al*. 2006).

Due to its transparency and the use of transgenic zebrafish expressing green fluorescent protein (GFP) in endothelial lineages, zebrafish is an excellent animal model to study tumor angiogenesis and metastatic behavior of transplanted tumor cells, showing all the critical steps of the metastatic process by live imaging at high resolution, including breaching of the basement membrane, intravasation, extravasation, and colonization of distant metastatic sites (Taylor & Zon 2009, Moore & Langenau 2012). The generation of the Casper mutant (Wenner 2009), which remain completely transparent throughout life, has provided to use xenograft tumor model also in juvenile/adult fish.

Original studies have shown the feasibility of injecting human melanoma cells in zebrafish embryos to follow their fate and to study their impact on host development. Tumor cells were injected into 3-h old zebrafish blastula-stage embryos to explore potential bidirectional interactions between cancer cells and embryonic cells. When injected at this early stage of development, highly aggressive melanoma cells survive but do not cause cancer or metastases, while they are able to redirect normal embryonic development, promoting formation of a secondary embryonic axis, probably due to Nodal signaling from the tumor cells (Lee *et al*. 2005, Topczewska *et al*. 2006). These results indicate that developing zebrafish can be used as a biosensor for tumor-derived signals. However, grafting of tumor cells at this stage, well before vascular development, results in their reprograming toward a nontumorigenic phenotype, thus hampering any attempt to investigate tumor-driven vascularization.

The first successful study on tumor-induced angiogenesis in zebrafish has been performed by Haldi *et al*. (2006). They reported that transplanted WM-266-4 melanoma cells into the yolk of zebrafish at 48 hours post fertilization (hpf) rapidly proliferated, migrated, formed tumor-like

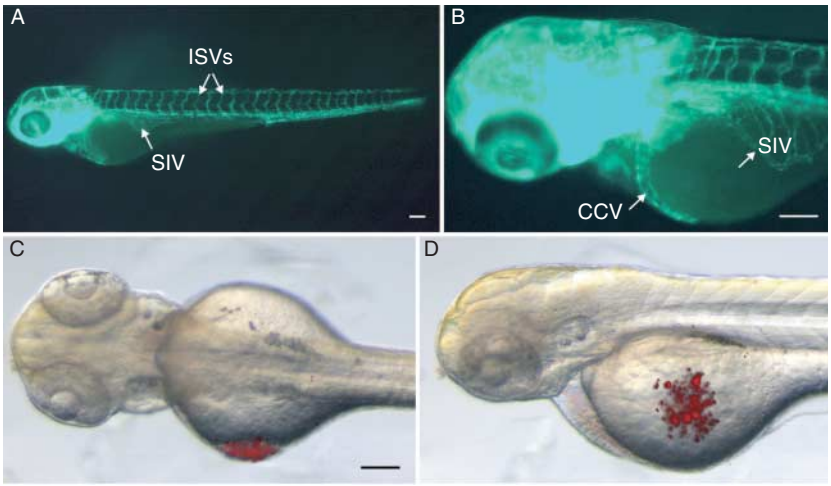

Figure 1

Human neuroendocrine tumor (NET) transplantation in zebrafish larvae. NET cells were injected in 48 hpf *Tg(fli1:EGFP)*^{y1} zebrafish larvae that expresses EGFP in the vascular endothelium (A and B). Red stained NET cells (by Celltracker Cm-DiI, Invitrogen) were grafted into the subepidermal space (between the periderm and the yolk syncytial layer) close to the SIV plexus (C: dorsal view, D: lateral view). Pictures were taken with light and fluorescent illumination and digitally superimposed. CCV, common cardinal vein; ISVs, intersomitic vessels; SIV, subintestinal vessels. Scale bar, 100 μm.

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masses, and stimulated angiogenesis through the recruitment of host endothelial cells and the formation of new vessels infiltrating the tumor mass.

Nicoli & Presta (2007) and Nicoli *et al*. (2007) demonstrated a potent angiogenic response triggered by mammalian tumor cells injected in the proximity of the developing SIV plexus in zebrafish embryos at 48 hpf. Pro-angiogenic factors released locally by the tumor graft, including fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), affect the normal developmental pattern of the SIV by stimulating the migration and growth of sprouting vessels toward the implant.

Marques *et al*. (2009) injected cells from gastrointestinal primary human tumors into the yolk sac of zebrafish embryos. Tumor cell invasion and micrometastasis formation were visible within 24 hours post-injection (hpi). Similar results were reported injecting highly metastatic murine melanoma B16–BL6 cells directly into the embryonic blood circulation in the ventral region of the duct of Cuvier. Tumor cells extravasated in different anatomical sites 24 hpi and formed extravascular micrometastases during the next 3–4 days (Tobia *et al*. 2013).

Stoletov *et al*. (2007) transplanted several human cancer cells into the peritoneal cavity of chemically immunosuppressed translucent zebrafish. Cancer cells expressing the metastatic gene *rhoC* employ an amoeboid-type invasion and stimulated angiogenesis. This system, taking advantage of the development of translucent fish and high-resolution confocal microscopy, provided the opportunity to visualize tumor invasion and metastasis in a model where mature fish vasculature mimics tumor-induced angiogenesis in human patients.

Very recently, Rampazzo *et al*. (2013) have injected glioblastoma multiforme (GBM) cells into the brain of developing zebrafish larvae. By using a Wnt-reporter zebrafish strain, they targeted primary human GBM cell injection into a Wnt-rich brain site and found that activation of Wnt signaling promotes neuronal differentiation of GBM cells, thus restraining GBM aggressiveness.

Therefore, when compared with other *in vivo* tumor angiogenesis/invasion/differentiation assays, this zebrafish/tumor xenograft model presents several relevant advantages which are as follows (Nicoli & Presta 2007, Tobia *et al*. 2011, 2013):

- Labeled tumor cells (e.g., GFP-transduced or fluorescent dye-loaded cells) can be easily visualized within the embryos, larvae, or Casper juvenile/adult fish. Because of the optical transparency and the availability of multiple zebrafish lines that express fluorescent proteins in normal tissues, zebrafish/tumor xenograft can provide a fast, high resolution on single-cell level and real-time monitoring of cell–stromal interactions and cancer progression in living animals (Konantz *et al*. 2012). The use of transgenic zebrafish, in which endothelial cells express GFP under the control of endothelial-specific promoters, represents an improvement of the zebrafish/tumor xenograft model, allowing the observation and time-lapse recording of newly formed blood vessels in live fish by epifluorescence microscopy as well as by *in vivo* confocal microscopy (Tobia *et al*. 2011). Several other available transgenic lines provide additional tools to study further aspects of the tumor–host interactions. For example, the use of transgenic zebrafish with neutrophils, macrophages, or platelets specifically labeled with fluorescent proteins, may improve our knowledge of the host inflammatory response against implanted tumors (Konantz *et al*. 2012, White *et al*. 2013).
- Immunohistochemistry and immunofluorescence staining can be performed on whole embryos and larvae or on histological sections to study protein expression and localization. Moreover, reverse transcriptase-PCR analysis with species-specific primers allows the concomitant study of gene expression by grafted tumor cells and by the host (Nicoli & Presta 2007, Tobia *et al*. 2011).
- Electron microscopy can be used in combination with light microscopy to perform detailed ultrastructural studies.
- As zebrafish at 48–72 hpf do not have a fully developed immune system, no graft rejection occurs at this stage. Therefore, the xenotransplantation procedure does not require immune suppression at this stage of development. Although, the main advantage to use juvenile/adult zebrafish compared with embryos is that all the major organs including the vasculature have completed development and have reached their mature pattern, at these stages zebrafish has a functional immune system that must be suppressed with dexamethasone or irradiation for successful grafting of the cancer cells (Tobia *et al*. 2011).
- Zebrafish embryos are readily permeable to many different compounds dissolved in their culture media. In this frame, the zebrafish/tumor xenograft model represents a rapid and suitable test to screen small-molecules with potential antitumor activity and using a small amount of compounds (Pichler *et al*. 2003). Interestingly, several groups recently have developed in zebrafish embryos quantitative,

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automated, and short-term bio-imaging platforms to study angiogenesis/cancer dissemination and for the screening of anticancer drugs (Vogt *et al*. 2009, Ghotra *et al*. 2012).
- The required low number of implanted cells (50–1000 cells/embryo) may favor the use of tumor cells isolated from human primary culture in order to perform drug sensitivity testing for personalized cancer therapy. In addition, the model allows the continuous delivery of angiogenic factors from a very limited number of cells, mimicking the initial stages of tumor angiogenesis and metastasis.
- Zebrafish are not expensive and can be easily maintained in an aquarium with a minimal requirement of equipment and propagated in a large number due to their high rate of fecundity (Mimeault & Batra 2013). The maintenance cost of zebrafish is considerably lower than that of mice (Pichler *et al*. 2003) and its logistic is much simpler than a mammalian facility.
- Transgenic reporter zebrafish lines can be used to track pathways involved in tumor–environment crosstalks (Moro *et al*. 2013, Rampazzo *et al*. 2013).

However, there are several disadvantages of using this model (Tobia *et al*. 2011, 2013), that need to be considered, such as:

- Species-specific microenvironmental differences may affect the behavior of grafted mammalian tumor cells and the lack of some mammalian organs in fishes (such as mammary gland, prostate, and lung) precludes the possibility to perform orthotopic transplantation experiments and to investigate tissue-specific mechanisms of tumor cell homing and colonization in these organs.
- Drug metabolism in zebrafish may be different from that in mammals.
- Zebrafish embryos are maintained at 28 °C. This may not represent an optimal temperature for mammalian cell growth and metabolism. However, the possibility to raise the incubation temperature up to 35 °C with no apparent gross effects on zebrafish development has been reported (Haldi *et al*. 2006).
- Embryonic organs and systems are completely defined but their differentiation is incomplete.
- A limited number of antibodies against zebrafish proteins are available so far. Nevertheless, due to the high degree of molecular conservation in vertebrates, antibodies that target mammalian protein can be used to perform immunohistochemistry and immunofluorescence assays on zebrafish samples.
- As for other animal models, xenotransplantation requires good manual skills of the operator.

Therefore, the zebrafish/tumor xenotransplantation is considered as an attractive, robust, fast, and technically simple model to study tumor–host microenvironment and to screen for antiangiogenic compounds.

Zebrafish as a cancer model for NETs

Most of the players, pathways, and feedback loops of endocrine system are highly conserved from zebrafish to human (Bourque & Houvras 2011, Lohr & Hammerschmidt 2011). Orthologs for several mammalian neurohormones have been identified and localized in zebrafish (Toro *et al*. 2009, Lohr & Hammerschmidt 2011). Therefore, the zebrafish is a relevant model for human endocrine system, providing important insights particularly into the development of endocrine glands (Porazzi *et al*. 2009).

Recent studies have suggested that zebrafish may emerge also as a new model of NETs with a reasonable prospect of success (Fig. 2).

Liu *et al*. (2011) generated a stable transgenic zebrafish (Tg:Pomc-Pttg) with overexpression of pituitary tumor transforming gene (*pttg*) targeted to the adenohypophyseal proopiomelanocortin (Pomc) cells. *PTTG* is overexpressed in more than 90% of pituitary tumors, including ACTH-secreting pituitary adenomas (Vlotides *et al*. 2007). Adult Tg:Pomc-Pttg fish developed pituitary corticotroph adenomas combined with pituitary cyclin E overexpression and metabolic disturbances, mimicking hypercortisolism caused by Cushing's disease. Although the chronic hypercortisolemic status was observed only in adult zebrafish, pituitary tumor was already detected within the first days of embryonic development. Like its mammalian counterpart, the Tg:Pomc-Pttg pituitary corticotroph adenoma developed cyclin E overexpression associated with G1/S phase disruption. This animal system has been adopted for an *in vivo* drug testing using several inhibitors of cyclin-dependent kinases (CDKs). R-roscovitine, a potent and selective inhibitor of CDK2/cyclin E, specifically reversed corticotroph expansion in live Tg:Pomc-Pttg embryos. This effect was subsequently confirmed in a mouse model of corticotroph (Liu *et al*. 2011).

Germline mutations of the aryl hydrocarbon receptor interacting protein (*AIP*) gene have been described in

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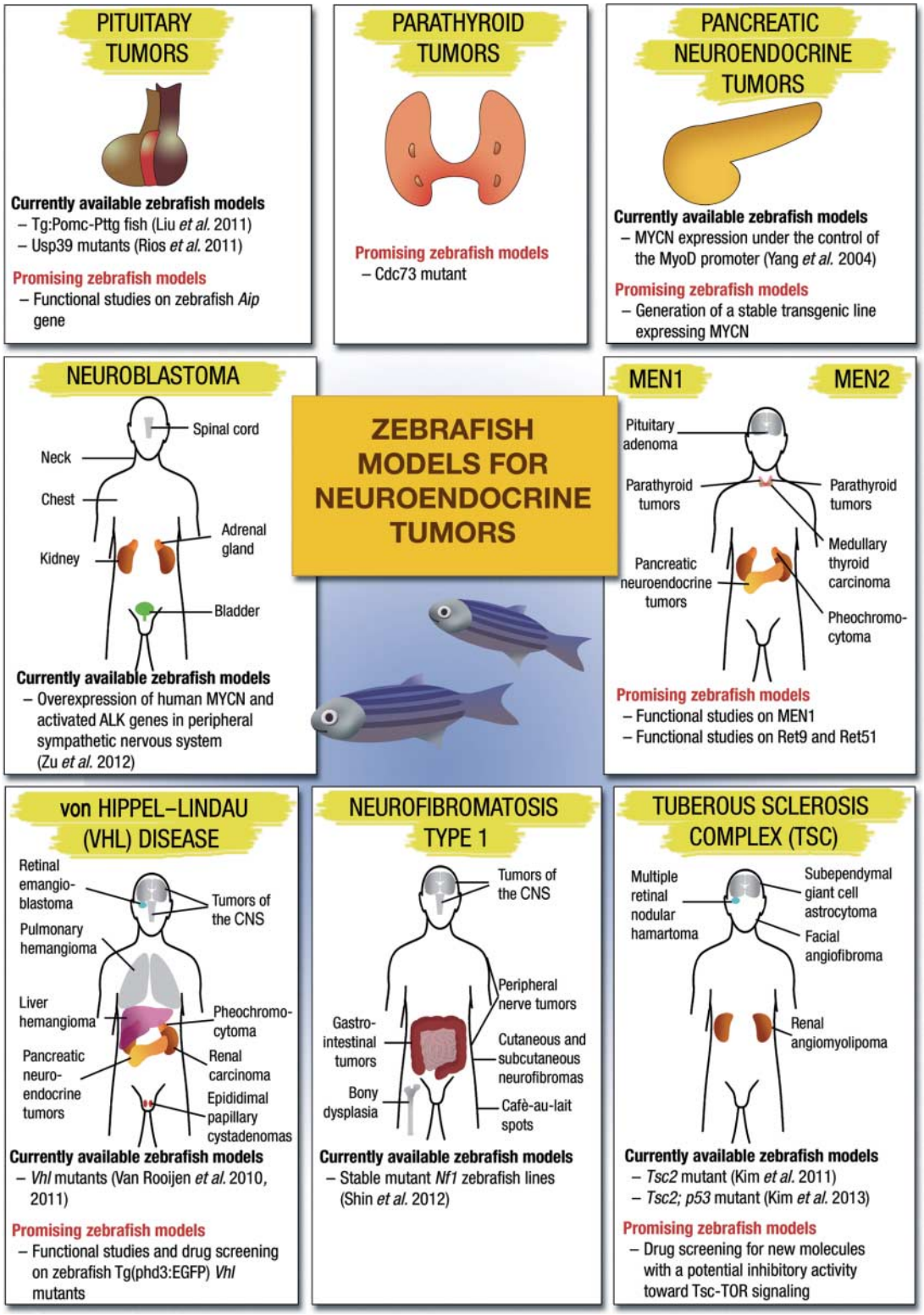

Figure 2
Currently available and promising zebrafish models to study neuroendocrine tumors (NETs).

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about 15–40% of familial cases of pituitary adenomas (Igreja *et al*. 2010). Equivalents of mammalian *AIP* are present and well conserved in the zebrafish. Studies on *aip* expression and functions in zebrafish are under investigation, offering a novel promising model to explore Aip protein interactions and to study pituitary tumorigenesis (Aflorei *et al*. 2012).

Other mechanisms potentially involved in pituitary tumorigenesis have been postulated through the use of the zebrafish model. By means of a forward genetic approach, Rios *et al*. (2011) identified a zebrafish ubiquitin-specific peptidase 39 (*Usp39*) mutant, developing a phenotype of microcephaly and pituitary hyperplasia. This study suggests that loss of *usp39* results in aberrant *retinoblastoma-1* mRNA splicing, which induces expression of its target *e2f4*, a transcription factor involved in controlling the cell cycle and with oncogenic activity when overexpressed. Indeed, gene expression profiling of *Usp39* mutants revealed a decrease in *retinoblastoma-1* and an increase in *e2f4*, *rbl2* (*p130*), and *cdkn1a* (*p21*) expression. These results disclose a new molecular mechanism, involving dysregulation of *retinoblastoma* and *e2f4* pathways, responsible for pituitary tumorigenesis.

Although fish do not have anatomical structures corresponding to parathyroid glands, they express parathyroid hormone and calcium sensing receptor in gill tissue, both of them are functionally similar to their mammalian counterparts. Indeed, parathyroid gland and the gills of fish are evolutionarily related structures (Bourque & Houvras 2011). In humans, germline inactivation of the *HRPT2/CDC73* tumor suppressor gene, coding for parafibromin and discovered in the context of the hyperparathyroidism–jaw tumor (HPT–JT) syndrome, has been reported in 50–75% of HPT–JT cases and in about 14% of familial isolated hyperparathyroidism (Carpten *et al*. 2002, Bricaire *et al*. 2013). In addition, *HRPT2/CDC73* mutation is a common, somatic event in most parathyroid cancers and adenomas, underlining the relevant role of this gene in the pathogenesis of parathyroid tumors (Sharretts *et al*. 2010). However, most of the mechanisms through which *HRPT2/CDC73* gene might control tissue-specific tumorigenesis are still unsolved. Interestingly, the zebrafish ortholog of *cdc73* has been identified in a genetic suppressor screen where it modulates erythropoiesis (Bai *et al*. 2010), and oligodendrocyte differentiation (Kim *et al*. 2012). The identification of a zebrafish *cdc73* mutant may provide an attractive device for creating a zebrafish model of parathyroid tumors (Bourque & Houvras 2011).

The potential role of surrounding tissue microenvironment in the pathogenesis of medullary thyroid cancer (MTC) may be investigated using zebrafish as model. In humans C-cells are dispersed throughout the thyroid parenchyma, whereas in zebrafish this cell type arises from the ultimobranchial bodies but does not come into contact with thyroid follicles. Malignant transformation of C-cells by *RET* oncogene leads to MTC in humans, but the role of surrounding follicular thyroid cells is presently unknown. So it would be interesting to determine the disease phenotype emerging in species where C-cells are not colocalized with thyroid epithelial cells (Bourque & Houvras 2011). Indeed, thyroid epithelial cells are able to synthesize extracellular matrix components in mammals, and it has been postulated that extracellular matrix may have a role in the pathogenesis and progression of MTC (Lekmine *et al*. 1999).

Zebrafish embryos represent an interesting model to study the factors and signaling events involved in pancreatic endocrine cell differentiation, proliferation, and carcinogenesis (Tehrani & Lin 2011). It is possible to perform chemical screens in transgenic zebrafish embryos aimed to identify compounds that modulate β-cell differentiation and proliferation (Hesselson *et al*. 2009, Rovira *et al*. 2011), providing determinant information to identify novel therapies for diabetes mellitus and pancreatic NETs. In this regard, when oncogenic human *MYCN* was expressed under the control of the zebrafish *myoD* promoter, that drives gene expression in pancreatic neuroendocrine β-cells, neurons, and muscle cells, a small number of the transgenic fish developed a neuroendocrine carcinomas between 4 and 6 months of age (Yang *et al*. 2004). It is well known that the *c-MYC* proto-oncogene is implicated in human pancreatic β-cells growth and tumorigenesis (Pelengaris & Khan 2001). This study suggested that *mycn*, a relative of *c-MYC*, may function in a similar manner in zebrafish (Yang *et al*. 2004). In future, the generation of a stable transgenic line expressing *MYCN* in the pancreas may provide a power drug-screening platform for pancreatic NET.

The *MYC/MAX/MXD1* network has also a critical role in the development of tumors of neural crest origin, such as neuroblastoma, pheochromocytomas, and paragangliomas (Cascon & Robledo 2012). Zhu *et al*. (2012) have generated a transgenic zebrafish model in which overexpression of human *MYCN* and activated anaplastic lymphoma kinase (*ALK*) genes in peripheral sympathetic nervous system develops tumors in the fish

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analog of the adrenal medulla that closely resemble human neuroblastoma.

With its genomic versatility and amenability to genetic and experimental manipulation, the zebrafish model may provide relevant insight into the study of hereditary disorders, including NETs as part of a hereditary syndrome.

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterized by the development of tumors of pituitary, parathyroid glands, and endocrine pancreas. The responsible gene *MEN1* encodes a 610-amino acid protein in humans, called menin. The gene is highly conserved in all vertebrate species including fish. Zebrafish menin is a 617-amino acid protein with 75% similarity to human menin and the region spanning residues 41–322 is highly conserved (83% homology). Amino acids affected by inactivating missense mutations in *MEN1* patients in this region are completely conserved between human and zebrafish. Such a high conservation strongly supports the functional relevance of this region (Khodaei *et al*. 1999). Analysis of the database of zebrafish mutants available from the zebrafish Information Network (http://zfin.org/action/fish/search) does not show any zebrafish *men1* mutant, but the generation of zebrafish mutants for this gene through the previously reported technologies may open novel interesting perspectives.

MEN2 is a hereditary disorder consisting of three syndromes: MEN2A, MEN2B, and familial MTC. These syndromes, due to germline-activating mutations of the *RET* proto-oncogene, result in the development of MTC and other tumors embryologically arising from the neural crest (Vitale *et al*. 2001). Human *RET* gene encodes two isoforms, termed *RET9* and *RET51*. Zebrafish *ret* is capable of encoding both isoforms. The zebrafish *ret9* amino acid sequence is identical to human *RET9*, and zebrafish *ret51* sequence shows significant sequence homology to human *RET51*, with 67% amino acid identity (Marcos-Gutierrez *et al*. 1997; Lucini *et al*. 2011). The exons encoding the tyrosine kinase domain are highly conserved from humans to zebrafish (Fisher *et al*. 2006). In zebrafish, *ret* signaling is crucial for the development of the enteric nervous system as in humans (Burzynski *et al*. 2009). Perturbation of *ret* and *gdnf* by morpholino knockdown resulted in a complete loss of the zebrafish enteric nervous system (Burzynski *et al*. 2009). In addition, neural crest cells can be directly visualized in live fish by using transgenic lines that express GFP in the enteric neurons, such as the FoxD3:GFP transgenic line (Field *et al*. 2009). Therefore, zebrafish represents an interesting genetic model to study Hirschsprung's disease, generally associated with lack of RET function (Burzynski *et al*. 2009). Newly developed *Ret* mutants in zebrafish, harboring activating mutations similar to those found in patients with MEN2, could provide relevant information toward understanding the mechanisms involved in this disease and could offer a powerful platform for drug screening.

A continuum of MEN is represented by the Von Hippel–Lindau (VHL) disease, an autosomal dominant genetic condition that results in a constellation of cysts and extensively vascularized tumors, including several NETs (pheochromocytomas and pancreatic NETs; Richard *et al*. 2013). Germline-inactivating mutations in the *VHL* gene cause this syndrome. The main function of *VHL* as tumor suppressor is to negatively regulate hypoxia-inducible mRNAs, including those encoding VEGF, erythropoietin, platelet-derived growth factor (PDGF), and glucose-transporter GLUT1. VHL is involved in the degradation by the proteasome of the hypoxia-inducible transcription factor HIF-1α. HIF-1α contributes to form transcriptional complex responsible for the activation of genes involved in angiogenesis, metabolism, and cell proliferation. In sum, the loss of VHL facilitates HIF accumulation that accounts for the excessive vascularization observed in VHL-related lesions and the development of tumors (Richard *et al*. 2013). In zebrafish the Vhl–Hif axis is highly conserved (Kajimura *et al*. 2006). *vhl* exhibits proangiogenic and tumor suppressor functions. Indeed, zebrafish *vhl* mutants develop several key aspects of the human disease condition, including activation of the Hif signaling pathway, severe pathological neovascularization, macular edema, pronephric abnormalities, and polycythemia (van Rooijen *et al*. 2010, 2011). Heterozygous *vhl* zebrafish, upon exposure to dimethylbenzanthracene, exhibited an increase in the occurrence of hepatic and intestinal tumors (Santhakumar *et al*. 2012). Interestingly, Vhl/Hif signaling can be evaluated *in vivo* in the zebrafish Tg(phd3::EGFP) line expressing enhanced GFP (EGFP) driven by prolyl hydroxylase 3 (phd3) promoter/regulatory elements. Since phd3 is strongly induced by the Vhl activation (Santhakumar *et al*. 2012), the expression of *vhl* mutants in the reporter zebrafish Tg(phd3::EGFP) line may represent a unique platform for the identification of new pathways involved in the development of VHL-associated neoplasms, including NETs. These models could be also helpful for chemical genetic screens aimed at identifying novel anti-angiogenic agents that are able to suppress HIF activity.

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Neurofibromatosis type 1 is a human genetic disorder characterized by café-au-lait macules and the growth of benign and malignant tumors involving the peripheral and CNS and NETs (pheochromocytoma, paragangliomas, gastroenteropancreatic-NETs). Inactivating mutations of *NF1* gene have been linked to neurofibromatosis type 1. Neurofibromin, the product of *NF1*, serves as a suppressor of the RAS activity (Laycock-van Spyk *et al.* 2011). Two zebrafish orthologs (*nf1a* and *nf1b*) are highly homologous to human *NF1* (about 84% identity). A zebrafish model of NF1 deficiency has been recently generated through stable mutant *nf1* zebrafish lines, using both zinc-finger nuclease and TILLING strategies (Shin *et al.* 2012). Zebrafish mutants lacking neurofibromin reveal abnormal patterning of the melanophores that compose the lateral stripes and are predisposed to tumor formation, a phenotype not very different from that reported in human neurofibromatosis type 1 (Shin *et al.* 2012). This zebrafish model represents an attractive tool to elucidate how NF1 mutations contribute to phenotypes and the mechanisms underlying the tissue-selectivity of tumors.

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder, characterized by the development of multiple hamartomas, and occasionally by NETs. This disorder is caused by loss-of-function mutations of the *TSC1* or the *TSC2* genes, which code for the proteins hamartin and tuberin respectively. Hamartin and tuberin constitute a tumor suppressor complex that negatively modulates mammalian target of rapamycin (mTOR) signaling, a critical pathway in the regulation of cell proliferation and angiogenesis in several tumors, notably in NETs (Dworakowska & Grossman 2009). Kim *et al.* (2011) developed a model system of TSC by introducing a premature stop codon in the zebrafish *tsc2* gene. *tsc2* homozygous mutant zebrafish exhibited several characteristics of TSC, including hamartoma formation in the brain and activation of TOR pathway (Kim *et al.* 2011). A similar model of TSC has been generated placing a heterozygous mutation of the *tsc2* gene in a *p53* mutant zebrafish. *tsc2*; *p53* mutants developed multiorgan malignancies with increased expression of Hif1-α, Hif2-α and Vegf-c, TOR activation and a conspicuous angiogenesis. Interestingly, mTOR inhibitor rapamycin significantly reduced tumor proliferation and vascularization (Kim *et al.* 2013). This zebrafish model would clarify most of the mechanisms contributing to tumorigenesis and mediated by dysregulation of the Tsc-TOR pathway. Another advantage of this model is its ability to accommodate large-scale anticancer drug screening for new molecules with a potential inhibitory activity toward Tsc-TOR signaling, representing a promising tool in the treatment of NETs.

The zebrafish/tumor xenograft angiogenesis assay in NETs: preliminary data

Angiogenesis has a critical role in the development of the tumor. Indeed, the formation of new vessels facilitates tumor metastasis and provides tumor cells with oxygen and nutrients, all essential factors to sustain the tumor growth. Most NETs have a highly diffuse vascularization. In fact, NETs typically produce a variety of proangiogenic cytokines and growth factors, including several members of VEGF, FGF, PDGF, epidermal growth factor (EGF), and insulin-like growth factor (IGF) families (Teule & Casanovas 2012, Scoazec 2013).

For the vast majority of tumors, the blood vessel density represents a prognostic indicator of survival and metastatic potential. In fact, tumors with high vascular density have a higher incidence of metastasis than poorly vascularized tumors. On the other hand, a paradoxical situation ('The neuroendocrine paradox') emerged in pancreatic NETs. In these tumors intratumoral microvascular density is higher in benign lesions than in carcinomas. Surprisingly, in malignant tumors microvascular density seems to be a favorable parameter, associated with a prolonged survival (Scoazec 2013). In addition, direct or indirect signs of proangiogenic response and hypoxia are expressed more clearly in high-grade than in low-grade tumors. To explain these observations, it has been postulated that in pancreatic NETs: i) the density of the vascular network is a marker of differentiation rather than a marker of aggressiveness; ii) angiogenesis is not tightly connected to metastatic properties. Therefore the most vascularized pancreatic NETs appear to be the most differentiated and the less angiogenic neoplasms (Scoazec 2013). In this regard, several issues need to be still addressed. As the 'neuroendocrine paradox' has been demonstrated only in pancreatic NETs, it remains to be verified whether it is translatable to the other types of NETs and to metastatic as well as to primary sites. These questions and a better knowledge of the mechanisms and regulation of tumor angiogenesis in NETs may be clinically highly relevant to determine the best anti-angiogenic therapeutic strategy.

As mechanisms playing a role in tumor–host interactions are highly conserved between human and

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zebrafish (Tobia *et al*. 2013), and the process of angiogenesis is mechanistically similar in embryonic and tumor development, we decided to perform the xenotransplantation of human NET cancer cells into the subperidermal space of zebrafish embryos (Fig. 1). It has been previously demonstrated that inoculation of mammalian tumor cells in zebrafish embryos can induce a potent angiogenic response through the secretion of several growth factors (Nicoli *et al*. 2007). VEGF/FGF gradient produced by the tumor is able to guide the

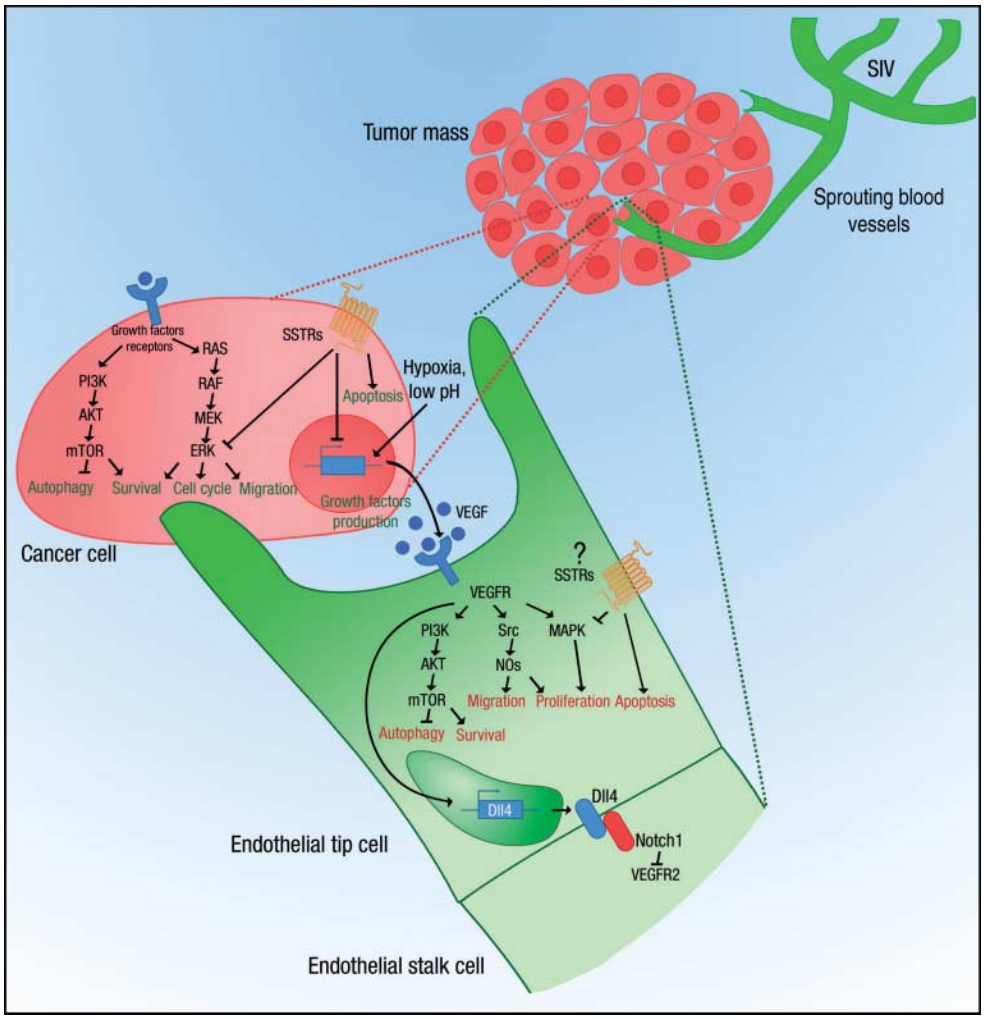

Figure 3

Schematic representation of putative molecular pathways involved in tumor xenograft-mediated angiogenesis. VEGF production is stimulated in implanted tumor cells by the hypoxia and low pH of the tumor microenvironment together with activation of different receptors for common growth factors (FGF, PDGF, EGF, IGF, etc.). These receptors promote several signal transduction events (Ras-Raf-MEK-ERK and PI3K-AKT-mTOR) that control cell cycle, survival, and migration of tumor cells. In addition, human VEGF secreted by the implant stimulates cell proliferation, migration, and survival of zebrafish endothelial cells probably through the activation of PI3K-AKT-mTOR, src-NOs, and MAPK. These processes induce and drive the sprouting of blood vessels from the subintestinal vessels (SIV) toward the tumor. The interplay between numerous signaling pathways provides an accurate phenotypic specialization of endothelial cells. A growing sprout consists of a tip cell that leads the developing vessel and extends philopodia during the migration and several stalk cells that form the vessel trunk. Molecular mechanisms that control the specification of tip and stalk cells are very conserved during the evolution of vertebrates and depend on the interaction between Notch and VEGF signaling (Siekmann & Lawson 2007). Hypoxia-driven VEGF signaling induces expression of the Notch ligand Delta-like-4 (Dll4) in tip cells. Then, the interaction between Dll4 and Notch receptor activates Notch pathway in adjacent endothelial cells, leading to the reduction of VEGF receptor 2 (VEGFR2) expression and thereby promoting the stalk cell phenotype (Siekmann & Lawson 2007). These processes are highly activated in neuroendocrine tumors and can be counteracted by the stimulation of somatostatin receptors (SSTRs) expressed in both neuroendocrine tumors cells and human endothelial cells of peritumoral vessels. SSTRs are conserved through evolution. However, the expression and the function of these receptors in peritumoral vessels need to be explored in zebrafish.

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sprouting of new blood vessels from the close vascular network (SIV). This is a complex phenomenon involving several pathways and mechanisms that are schematically illustrated in Fig. 3. Interestingly, most of these pathways deregulated in zebrafish/tumor xenograft model are commonly activated in human NETs.

We have recently developed a system to study NET-mediated angiogenesis (Vitale G, Gaudenzi G, Dicitore A, Cotelli F and Persani, 2013, unpublished observations), based on the injection of two human NET cell lines (TT, a human MTC cell line and DMS79, a human small-cell lung carcinoma cell line secreting ACTH) in *Tg(fli1:EGFP)*^{y1} zebrafish line that expresses EGFP under the control of the *fli1* promoter (Fig. 1A and B). Both NET cell lines have been selected on the basis of strong proangiogenic capacity, related to the high production of VEGF (Lund *et al*. 2000, Petrangolini *et al*. 2006).

Starting from 24 hpi, we evaluated the ability of both tumor cell lines to induce the sprouting of new vessels from the SIV and the CCV (Fig. 4). While the control larvae injected with only phosphate buffered saline solution (PBS) did not display alterations of vascular network, the injection of TT and DMS79 cells line stimulated migration and growth of sprouting vessels from SIV and CCV toward grafted cells in a time-dependent manner (Fig. 4). Indeed, we observed new blood vessels that rapidly reached the graft and progressively surrounded and penetrated the tumor cells mass. A more intricate network of new blood vessels was observed in TT tumor-xenograft (Figs 4 and 5). In a temporal window of three days post injection, we observed that the neovascularization followed morphogenetic steps resembling physiological angiogenesis that occurs during embryonic development and in adult animals (Fig. 5A, B, C, D, E, and F). Indeed, in 24 and 48 hpi TT-grafted larvae we detected that endothelial cells leading the growing sprout have a 'tip phenotype', with long philopodia that probably explore molecular signals in the microenvironment of tumor cells (Fig. 5A, B, D, D′, E, and E′). Moreover, we observed that endothelial sprouts with tip cells were progressively converted in vessels (Fig. 5C, F, and F′) (Adams & Alitalo 2007). Histological sections of 48 hpi TT-grafted larvae stained with whole-mount alkaline phosphatase clearly showed that new vessels reached and penetrated the tumor mass (Fig. 5G, H, I, and J).

Somatostatin and dopamine receptors, as well as mTOR pathway, represent pivotal controllers of hormonal secretion, cell proliferation, and angiogenesis in human NETs (Gatto & Hofland 2011). Indeed, somatostatin analogs, dopamine agonists, and mTOR inhibitors are

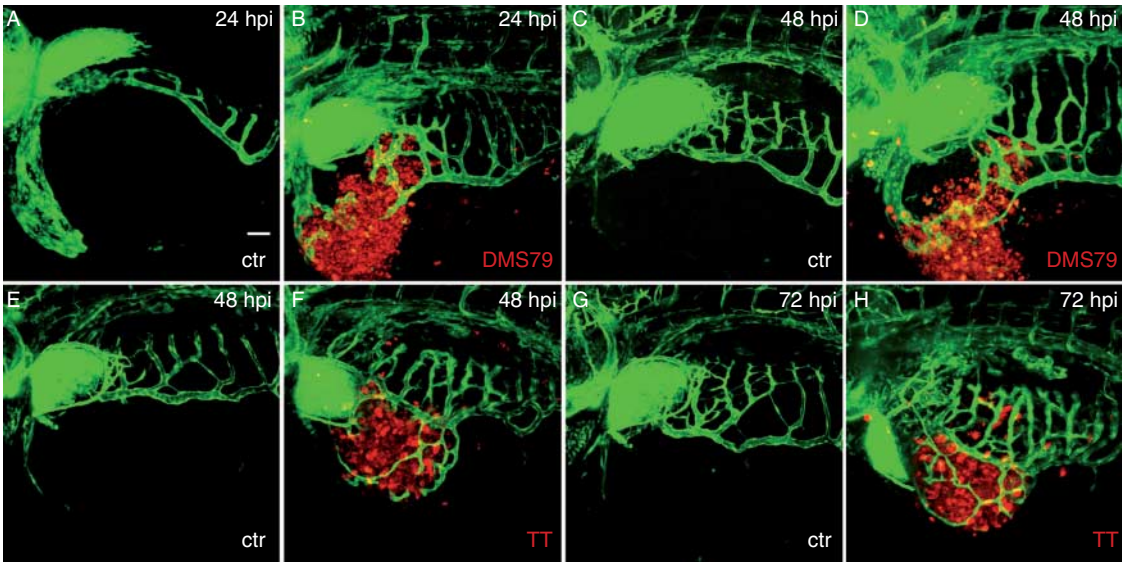

Figure 4

Neuroendocrine tumor (NET)-grafted cells stimulate angiogenesis in zebrafish larvae. Representative confocal microscopic images of 48 hpf *Tg(fli1:EGFP)*^{y1} zebrafish larvae implanted with red fluorescence-stained DMS79 (B and D) and TT (F and H) cells. After 24 (A and B), 48 (C, D, E, and F), and 72 hpi (G and H) larvae were embedded in low-melting agarose and the yolk region was observed by confocal microscopy. In comparison to PBS-injected control larvae (A, C, E, and G), NET-grafted larvae showed vessels that sprout from the SIV and the CCV (B, D, F, and H). TT seemed to have a more robust proangiogenic activity (F and H). All images are oriented so that rostral is to the left and dorsal is at the top. Scale bar, 50 μm.

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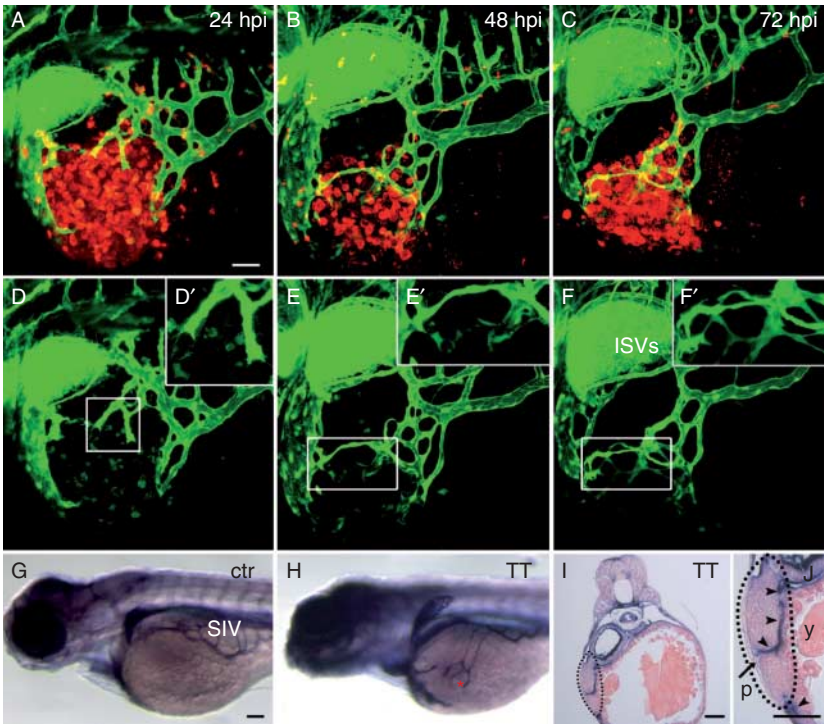

Figure 5

Progressive vascularization of tumor cells mass in TT tumor xenografts. Representative microscopic images of 48 hpf *Tg(fli1:EGFP)*^{y1} zebrafish larvae implanted with red fluorescence stained TT cells. The same TT grafted larva was observed by confocal microscopy (A, B, C, D, D′, E, E′, F, and F′) at 24 (A and D), 48 (B and E), and 72 (C and F) hpi. Images in A, B, and C showed sprouting vessels that progressively reached and surrounded red grafted cells. The red channel image was omitted in panels D, D′, E, E′, and F to highlight the newly formed microvascular network. Digital magnification of boxed regions in D, E, and F (D′, E′ and F′) suggested that tumor xenograft mediated angiogenesis is a multistep process, in which endothelial sprouts with tip cells (cells with long philopodia in D′ and E′) were progressively converted in vessels. Alkaline phosphatase staining was performed on 48 hpi control (G) and TT grafted larvae (H, red asterisk indicates the position of TT cells). Transverse histological sections (I and J) of alkaline phosphatase-stained TT-grafted larvae displayed that vessels penetrated in the tumor mass. Dashed areas in I and J (original magnification of tumor mass) showed the position of tumor cells between the periderm (p) and the yolk (y). Arrowheads in J indicate neoformed vessels surrounding and penetrating the tumor implant. Images from A to H are oriented so that rostral is to the left and dorsal is at the top. Scale bar, 50 μm.

currently used in the therapy of NETs (Faggiano *et al*. 2012, Ruscica *et al*. 2013).

Mammals have five somatostatin receptor genes, named SSTR1 through 5 (Olias *et al*. 2004), whereas zebrafish has eight SSTR genes: SSTR1, -2a, -2b, -3a, -3b, -5a, -5b, and -6 (Ocampo Daza *et al*. 2012). Comparative genomic analyses suggested that SSTRs family arose from a series of gene duplication events throughout the course of vertebrate evolution. In particular, the increase in SSTRs family members could be the result of the basal vertebrate whole-genome duplications and subsequently the teleost-specific genome duplication. One of the teleost receptors gene, *sstr6*, represents an ancestral vertebrate subtype that has been lost in tetrapods, while *sstr4* sequences could not be identified in teleosts (Ocampo Daza *et al*. 2012). Zebrafish and human amino acidic SSTR sequences showed a high degree of identity ranging 50–80%.

In mammals, five-specific dopamine receptors have been characterized and are classified into two subgroups: D1-like (D1, D5) and D2-like receptors (D2, D3, D4) (Ferone *et al*. 2009). In zebrafish, 8 dopamine receptors have been cloned (Barreto-Valer *et al*. 2012): D1-like receptor (Drd1), which shares 71% amino acid identity to humans (Li *et al*. 2007); the D2-like receptors (Drd2a, Drd2b, Drd2c, Drd3, Drd4a, Drd4b, and Drd4rs), which show an amino acid identity with human sequence ranging 56–67% (Boehmler *et al*. 2004, 2007).

Like its mammalian counterpart, the zebrafish TOR ortholog (zTOR) plays a central role in the regulation of cell proliferation and angiogenesis. Indeed, *TOR* is a highly conserved serine–threonine kinase that is a physiological target of embryonic growth-associated protein (EGAP) N-terminal acetyltransferase complex during zebrafish development. Its role into angiogenesis is supported by

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several experimental evidences. Indeed, pharmacological inhibition of TOR with rapamycin leads to growth and vessel defects resembling the phenotypes of EGAP knockdown. Moreover, the overexpression of constitutively active TOR rescued normal vessel phenotype (Wenzlau *et al*. 2006).

Therefore, the zebrafish model may be exploited to investigate the molecular mechanisms underlying the SSTR-, dopamine receptor-, and TOR-dependent inhibition of NET tumor angiogenesis.

Conclusions

Only few models are currently available for NETs, probably due to the rare occurrence and heterogeneity of this group of neoplasms. These have been mainly developed in rodents and have been useful to understand the role of oncogenes or tumor suppressor genes involved in the development of various types of NETs (Pellegata *et al*. 2006). More recently another interesting NET model includes three-dimensional cell culture, a valuable method for drug screening due to its relevance in modeling the *in vivo* tumor size organization and microenvironment.

In this frame, our zebrafish/NET xenograft model may represent an attractive, fast, and technically simple model to study tumor–host microenvironment, to better characterize the multiple mechanisms of angiogenesis in NETs and to test *in vivo* the effects of new compounds (such as somatostatin–dopamine chimeras, dual PI3K/AKT/mTOR inhibitors, tyrosine–kinase inhibitors) on tumor angiogenesis. In addition, this model can potentially provide an exhaustive response to the unanswered questions related to the 'neuroendocrine paradox'.

In conclusion, there is reasonable hope that zebrafish can represent an optimal experimental model in NETs for drug screening and to elucidate molecular mechanisms involved in tumorigenesis and cancer progression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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