Transplantable zebrafish models of neuroendocrine tumors

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Abstract

Neuroendocrine tumors (NETs) are rare neoplasms whose incidence is increasing. NETs constitute a heterogeneous group of tumors. Their clinical features, functional properties, and clinical course are different on the basis of their site of origin. Due to the heterogeneity of these tumors, a coordinated multidisciplinary approach is required in these patients. However, medical doctor encounters many difficulties when providing care for patients with NETs. This review provides an overview of the state of the art of zebrafish model in the cancer research with a main focus on NETs.

Résumé

Les tumeurs neuro-endocrines (TNE) sont des néoplasmes rares dont l'incidence est en augmentation. Les TNE constituent un groupe hétérogène de tumeurs avec caractéristiques cliniques, propriétés sécrétoires et fonctionnelles et évolution qui varient selon leur site de développement initial. En raison de l'hétérogénéité tumorale, il est necessaire d'adopter une approche multidisciplinaire pour la prise en charge de ces patients. Toutefois, les difficultés rencontrées par les médecins lorsque la prise en charge d'un patient atteint par un TNE sont nombreuses. Cette revue vise à analyser le potentiel du poisson zèbre dans la recherche sur le cancer, en portant une attention particulière aux TNE.

Introduction

Neuroendocrine tumors (NETs) are a class of rare and heterogeneous neoplasms derived from the neuroendocrine system. The term "neuroendocrine" is applied to widely dispersed cells with "neuro" and "endocrine" differentiation and properties. Due to the body-wide distribution of these cells, NETs can arise in several anatomic sites. The most common primary tumor sites are the gastroenteropancreatic tract and lungs (1). The biological behavior and clinical characteristics of NETs vary considerably. In addition, most of the standard treatments lead to the development of drug-resistance or undesirable adverse effects and have limited success rates. Because of the substantial heterogeneity in the clinical aggressiveness and the responses to therapy of these neoplasms, the optimal treatment selection for patients represents a clinically difficult challenge (2-4).

In the last decades considerable efforts have been made to develop a valid classification and grading system in order to better categorize NETs according to their clinical behavior and with respect to impact on treatment and prognosis (5-7). However, given the heterogeneity of these tumors the therapeutic decision-making is extremely difficult, and it requires a coordinated multidisciplinary approach involving surgeons, endocrinologists, oncologists, gastroenterologists, radiologists, pathologists, radiation oncologists, nuclear medicine specialists, genetic counselors, etc (8, 9). Through this strategy a personalization of the therapy is used, but managing criteria are still far from those that underlie the most recent applications of personalized medicine in Oncology. This emerging paradigm for the clinical oncology is focused on tailoring the optimal drug for each individual patient, not empirically on the basis of the clinical–pathological

profile, but taking advantage of individual prognostic and predictive biomarkers or evaluating the effects of several candidate drugs in patient-derived preclinical models (10, 11).

Patient derived xenograft (PDX) model in oncology: pros and cons

In the last decade, 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. This platform has been widely used for preclinical drug-screening and many evidences support its use to predict the individual response to cancer treatment (10-13). PDX procedure is based on transplanting primary tumor cells, obtained from surgery or from a biopsy, into an immunocompromised animal. Implanted mass consists in tissue fragment or single-cell suspension, isolated after chemically digestion or physically manipulation. The most common used immunodeficient mice strains are SCID, NOD-SCID, NSG, and athymic nude mice. The tumor implantation could be performed heterotopically or orthotopically. Heterotopic PDX models consist in implanting cancer cells into an area of the mouse other than that of the original tumor site, generally into the subcutaneous flank of the animal. This method is technically simple and optimal for an easy monitoring of tumor growth. Orthotopic model involves the implantation of the patient's tumor tissue into the corresponding anatomical organ of the animal. This method is more technically difficult, but it can reproduce the natural environment. This aspect could be relevant for the optimal growing of the tumor. Indeed, when a tumor is heterotopically transplanted into a mouse subcutaneously it often does not grow and metastasize as expected in a patient

(10-13). While, orthotopic PDX models showed increased incidence of metastases from transplanted tumors, compared with heterotopic subcutaneous models (14). PDX takes about 2 to 4 months for the tumor to engraft, depending on the tumor features (histotype, stage, grade, and aggressiveness), implant location and strain of mice. Upon engraftment and when the tumour burden becomes too high, the tumour is harvested and prepared for transplantation into several animals to develop a mice cohort of PDX that could be used for preclinical experiments (13).

One of the most intriguing applications of PDXs is their use in decision-making, to identify the most appropriate and personalized therapy. For example, PDXs have been recently used in co-clinical studies, in which patient-derived tumor cells are implanted into immunocompromised mice, subsequently treated with the same drug or the same combination therapy that the patient receives in the study. PDX-based co-clinical studies, integrating preclinical and clinical data in real time, offers the unique possibility to personalize the therapy at the time of disease progression, eventually identifying more quickly strategies to overcome resistance to the treatment (15).

Although murine PDX model remains the gold standard, this model has relevant limitations: it usually takes several months to have a visible tumor implant, this could be too long for cancer treatment decision-making processes; the immune system is compromised in the recipient mice in order to avoid transplant rejection, this may limit predictive value for a specific therapeutic approach and animals are more susceptible to infection and drug toxicity than normal mice; its laborious and time-consuming process makes this model very expensive; large number of cells (about 1 million) are required to generate a tumor; high difficulties to generate

mouse xenotransplant models able to metastasize (12, 13). In NETs these limitations are further complicated by the limited availability of tumour cells (because of the small size of post-surgical samples) and the extremely low engraftment rate of these in mice (because of the slow tumour growth).

In the frame of developing new preclinical in vivo models for NETs, our group have exploited the zebrafish, an attractive human disease model, particularly for cancer research.

Introduction to Zebrafish as a cancer model

The telostean zebrafish (*Danio rerio*) is a popular vertebrate model system that offers unique advantages for the study of a variety of biological processes. The small size and low cost of maintenance, together with the high prolific nature, the external fertilization and the rapid development of its transparent embryos have led to the first emergence of the zebrafish as reliable and suitable embryological model. Moreover, due to the proved conservation in genetic programs and physiology between fish and mammals, zebrafish has become a powerful model for studying human diseases, including cancer (16).

Zebrafish can develop tumors in various organs with high degree of histological and molecular conservation compared with human malignances (17, 18). It has been shown that many aspects of human cancer, comprising both cancerogenesis and tumor progression, can be recapitulated in zebrafish, offering a unique opportunity for exploiting molecular mechanism underlying this disease and testing new pharmacological strategies (16, 19).

Several strategies have been used to generate cancer models in zebrafish. Initial studies relied on tumor induction through chemical carcinogens, added into the fish water or administered by injection or food. Chemical carcinogens have been successfully used in toxicological studies, in forward genetic screens or in combination with genetic manipulation in defining a tumor susceptibility phenotype (20-23). Although the exposure of zebrafish to different carcinogens can induce the formation of a wide variety of neoplasms (24, 25), it has been reported that tumor incidence is generally low, tumor induction is not organ-specific and more frequently regards liver (26). Another approach to study cancer in zebrafish consists in the generation of mutant and transgenic lines as gene-based tumor models. Zebrafish lines harboring inactivating mutations in tumor-suppressor genes (e.g. *tp53*, *apc*, and *pten*) have been identified in forward genetic screens or generated by several technologies, such as Zinc finger endonucleases (27), TALENs (28) and CRISPR/Cas9 (29), that induce local lesions in zebrafish genome (20, 30, 31). A representative example is the *tp53*^{M214K} mutant line, harboring a missense mutation in *tp53* tumor-suppressor gene. During development, *tp53*^{M214K} embryos failed to undergo apoptosis in response to γ radiation and, starting from at 8.5 months of age, 28% of mutants developed malignant peripheral nerve sheath tumors (30). In addition, several zebrafish transgenic lines have been developed with oncogenes under the control of tissue specific promoters. For instance, the stable expression of BRAF^{V600E}, the most common mutation in melanoma, under the control of the melanocytes-specific promoter *Mitfa*, induced the development of melanocyte lesions, histologically resembling human nevi. Interestingly, when BRAF^{V600E} was expressed in the

melanocytes of *tp53*^{M214K} fish, some of the fish nevi progressed to melanoma by the time (32). Transgenic lines have been generated to create disease models not only of solid tumors but also of lymphoid neoplasms. In this context, the first stable transgenic line of T-cells acute lymphoblastic leukemia was obtained by expressing *Myc* oncogene in zebrafish T-cells (33).

Another strategy to study cancer in zebrafish is based on the xenotransplantation of human or mouse cancer cells in several sites of embryos, larvae, juvenile and adult fish. Due to intrinsic features of zebrafish embryos, together with the availability of transgenic lines that express fluorescent proteins in normal tissues, tumor xenograft in embryos currently represents the most used transplantable zebrafish platform to rapidly monitor cell–stromal interactions, tumor-induced angiogenesis and migration of implanted tumor cells (34). Although murine xenotransplantable model remains the gold standard, tumor xenograft in zebrafish embryos can overcome some drawbacks, previously reported in mice. For instance, maintenance cost of zebrafish model is lower than that of mice and its logistic is much simpler than a mammalian facility. All observations on grafted embryos can be made *in vivo* and the response to tumor implantation is faster than in mice. Indeed, proangiogenic effects of implanted cells or their metastatic behavior can be observed only after 24 hours post injection (hpi). As 48–72 hours post fertilization (hpf) zebrafish embryos do not have a fully developed immune system, no rejection occurs, thus immunosuppression is not needed as in mouse model.

Zebrafish embryos have been used as recipient for xenotransplantation assays with both immortalized cell lines and primary culture generated from post-surgical

tumor samples. In this context, zebrafish PDXs have been recently suggested as promising platform for the development of precision medicine applications, due to the high potential in predicting the individual response to anticancer treatments. In particular, a recent study has demonstrated that PDXs of human colon rectal cancer in zebrafish respond to the available therapeutic options, present in the international guidelines, as in human patients (35).

NET transplantable models in zebrafish embryos

In order to develop a preclinical platform for the study of NETs, our group has firstly set up a system based on the injection of several human NET cell lines in the subperidermal cavity of *Tg(fli1a:EGFP)*^{y1} zebrafish embryos (36), that expresses EGFP in the entire vascular tree under the control of the endothelial *fli1a* promoter. We demonstrated that this platform is very useful to quickly analyze *in vivo* the proangiogenic potential of implanted NET cell lines (37). Indeed, the presence of grafted cells affected the physiological angiogenesis of both sub intestinal vein (SIV) plexus and the common cardinal vein (CCV), leading to the formation of endothelial sprouts in only 24 hpi, that progressively converted in vessels in the next time window of 48 hours.

More recently, we have set up a procedure, based on the injection of patient-derived NET tumor cells in zebrafish embryos, that offers unique advantages to evaluate *in vivo* two relevant aspects linked to the individual tumor progression, such as tumor-induced angiogenesis and invasiveness (38). Post-surgical samples of several NET patients were firstly used to generate primary cultures. Then, NET cells were stained with a fluorescent dye into the subperidermal cavity of

Tg(fli1a:EGFP)^{y1} zebrafish embryos. While control embryos did not display alterations of vascular network, grafted embryos showed vessels that sprout from the subintestinal vein plexus toward the tumor mass after only 24 hpi. At the same time, grafted NET cells showed a strong invasive behavior, migrating out from the tumor mass at the injection site and invading different parts of the embryo, in particular the area of the posterior caudal vein plexus (figure 1). Moreover, we demonstrated that injected NET cells preserved nuclear morphology and the expression of specific molecular markers (38-40).

Due to the possibility to study the effects of small tumor implants (100 cells/embryo), the platform resulted particularly suitable for NETs, where the post-surgical availability of tumor cells is often limited. Moreover, the success of transplantation in zebrafish embryos resulted to be extra ordinary higher compared to that reported for PDX murine model (41, 42).

Together with the possibility to better investigate the tumor–host microenvironment, angiogenesis and invasiveness, NET xenografts represent an advantageous platform to test new anticancer molecules, due to the versatility of zebrafish embryos in drug screenings. Indeed, 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 blood circulation (37).

Conclusions

In the field of NETs, where only few preclinical models are currently available, zebrafish xenograft model seems to be an innovative tool to study the tumor–host

microenvironment and drug discovery. NET PDXs in zebrafish embryos may contribute to the development of new precision medicine applications, predicting the individual clinical response to novel compounds or to a combination of those. Moreover, they could be helpful to identify the correct sequence of treatment in each individual NET patient, finalized to a better efficacy and less toxicity. This strategy could be relevant to improve survival and quality of life in patients with NETs.

Declaration of interest

The authors declare that they have no competing interest.

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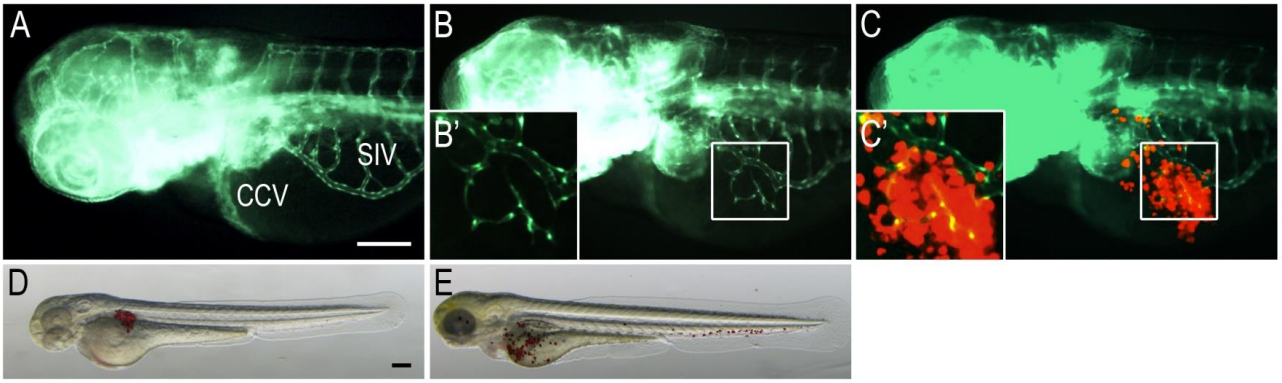
Figure legend

Figure 1. Tumor induced angiogenesis and tumor cell migration in zebrafish embryos grafted with primary culture cells derived from a patient with NET. Red stained NET cells (by Celltracker CM-DiI, Invitrogen) were grafted into the subperidermal space (between the periderm and the yolk syncytial layer) close to the SIV plexus of 48 hours post fertilization *tg(fli1a:EGFP)^{y1}* zebrafish embryos, that express EGFP in vascular endothelium. After the injection, embryos showing cells into the yolk sac and/or in the vasculature were discarded. At 24 hpi, while PBS-injected control embryos (A) showed a normal development of SIV plexus, PDX embryos (B, C) showed endothelial structures (green) that sprouted from the SIV. In panel B the red channel has been omitted to highlight the tumor-induced angiogenic sprouts. Panels B' and C' represent a digital magnification of boxed regions in B and C, respectively. The spread throughout the body of stained tumor cells was evaluated comparing their localization soon after the implantation (D), when tumor cells were well-confined at the injection site, and 24 hpi (E), when cells were detected in distant areas, such as the head or the posterior caudal vein plexus. Representative fluorescent and bright-field images were merged in D and E. All images are oriented so that rostral is to the left and dorsal is at the top. CCV, common cardinal vein; SIV, subintestinal vessels. Scale bars in A and D, 100 µm.

Figure 1. Angiogenèse tumorale et migration de cellules tumorales dans les embryons de poisson zèbre, greffés avec des cellules d'un patient atteint d'un tumeur neuroendocrine. Des cellules d'un tumeur neuroendocrine, marquées en

rouge (par Celltracker CM-DiI, Invitrogen), ont été greffées dans l'espace sous-péridermique (entre le périderme et la membrane qui enveloppe le jaune), près du plexus de la SIV, des embryons tg (fli1a: EGFP) y1 au stade de 48 heures après la fécondation, qui ont vasculature fluorescent. Après la grèffe, les embryons présentant des cellules dans le sac vitellin et / ou dans le système vasculaire ont été jetés. À 24 heures après la fécondation, les embryons de control injectés avec du PBS (A) présentaient un développement normal du plexus. Au contraire, les embryons xénogreffés (B, C) présentaient des structures endothéliales (vert) qui naissaient de la SIV. Dans le panneau B, le canal rouge a été omis pour mettre en évidence les germes angiogéniques induits par la tumeur. Les panneaux B 'et C' représentent un agrandissement numérique des régions encadrées dans B et C, respectivement. La propagation des cellules tumorales a été évaluée en comparant leur localisation peu après l'implantation (D), lorsque les cellules tumorales étaient bien confinées au site d'injection, et à 24 hpi (E), lorsque les cellules ont été détectées dans des zones éloignées telles que la tête ou le plexus de la veine caudale postérieure. Des images représentatives des champs fluorescents et lumineux ont été fusionnées en D et E. Toutes les images sont orientées de sorte que le rostral soit à gauche et le dorsal en haut. CCV, veine cardinale commune; SIV, vaisseaux sous-intestinaux. Barres d'échelle en A et D, 100 µm.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