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Zebrafish research in Greece: swimming against the current

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Abbreviations: bdnf, brain-derived neurotrophic factor; BrdU, Bromodeoxyuridine; BRFAA, Biomedical Research Foundation of the Academy of Athens; crf (corticotropin-releasing factor); DDX3X, DEAD-Box Helicase 3 X-Linked; DYNC1I2, Dynein Cytoplasmic 1 Intermediate Chain 2; GWAS, Genome-Wide Association Studies; hpf, hours post fertilization; kdrl, kinase insert domain receptor like; myh6, myosin heavy chain 6; NGS, next-generation sequencing; prkd2, protein kinase d2; ProTα, Prothymosin alpha; SET/I2PP2A, protein phosphatase 2A inhibitor 2; sGC, soluble Guanylyl Cyclase; spim, Spinning Disc Microscopy; TGFβ, transforming growth factor beta; VEGFR2, vascular endothelial growth factor receptor-2; UAS, upstream activated sequence; zPDX, Zebrafish patient-derived xenografts;
Abstract

Zebrafish is a vertebrate model extensively used in Developmental Biology and Human Disease modeling as it shares high genetic and physiological similarities with humans. It has become the second most popular animal model, following mice, with several advantages: zebrafish are easily housed and cared for, the cost of installing and maintaining a zebrafish facility is significantly lower than mice, they reproduce often and develop quickly. Using zebrafish complies with the 3Rs principles of laboratory animal use. Zebrafish embryos develop externally and are transparent, allowing for in vivo non-invasive imaging. There are many transgenic and mutant lines available that mimic most Human Diseases, including reporter lines for most signaling pathways. There are also several reverse genetic tools to functionally verify genes or variants of unknown significance, identified in Genome-Wide Association Studies (GWAS) or using Next Generation Sequencing (NGS) approaches. In addition, the model emerges as an invaluable whole animal platform for various stages of drug discovery efforts by exploring the ability to do high-throughput phenotypic-driven screens. These include phenotypic screenings, determinations of general and/or specific toxicity (cardiac, renal, hepatotoxicity etc.), and mechanism of action studies. Finally, zebrafish are able to retain their capacity to regenerate most organs during their entire life span, making them a well-established model to study organ regeneration. The European Zebrafish Society consists of more than 180 research labs throughout Europe. In Greece however, zebrafish use remains rather limited. I present here a brief historical overview of zebrafish research in Greece.
**Zebralish in Developmental Biology**

The extraordinary ability to observe development non-invasively always amazes somebody that first looks through a stereoscope at a zebrafish embryo. The advances of microscopy and, most importantly, of the Spinning Disc Microscopy (spim) in combination with transgenic lines was pivotal in enabling the detection, imaging and reconstruction of development and organogenesis at cellular resolution (Huiskens et al., 2004; Keller et al., 2008). Advances in next-generation sequencing (NGS) techniques gave the ability to reconstruct a transcriptomics-based single-cell lineage of development and organogenesis (Wagner et al., 2018; Farrell et al., 2018).

Looking at live embryos and, more specifically, their beating hearts is how I got hooked on zebrafish research. After my post-doctoral training at the University of California San Francisco (UCSF), where I participated in a forward genetic screen (Beis et al., 2005), I decided to move back to Greece and set up my own zebrafish facility at the Biomedical Research Foundation of the Academy of Athens (BRFAA) in 2005 (Figure 1). At that time with the team of the Stainier lab I have just experienced the relocation and reinstallation of a large zebrafish facility from the Parnassus campus to Mission Bay, so moving some fish across the ocean did not seem such a challenge.

The first years after establishing my lab in BRFAA, we primarily focused on Cellular and Developmental Biomedical approaches to Cardiovascular Development and Disease. To my knowledge, I was the first to introduce zebrafish Biomedical Research in Greece and still maintain the largest housing facility. The ease in studying cardiovascular development and angiogenesis in vivo was quickly appreciated and in 2006, we showed that activation of soluble Guanylyl Cyclase (sGC) promotes angiogenesis in a first collaborative paper with greek research teams. (Pyriochou et al., 2006). Since then, we have extensively used the Tg(kdrl:eGFP) line to screen for angiogenesis inhibitors in several projects. We used it to select molecules with the maximal activity of VEGFR2 inhibition (Papakyriakou et al., 2014) and peptid conjugated molecules synthesized to target cancer cells (Argyros et al., 2018).

**Zebralish models of Cardiovascular Development and Disease**

The zebrafish heart provides a powerful in vivo system to study cardiac development and regeneration at the cellular level (Bournele and Beis 2016; Giardoglu and Beis 2019). We used cardiac valve development to dissect the role of intracardiac flow dynamics in mutants with unlooped hearts (southpaw) and non contracting atrium (myh6, weak atrium) (Kalogirou et al., 2014). The fascinating phenotype of myh6 adults that survive with a single functioning chamber allowed us to study a model of adult ventricular remodeling. We showed that the increased ventricular size is due to a hyperplastic response, besides exhibiting a transcriptional program that in mammals is mostly associated with hypertrophy (Sarantis et al., 2019).

Currently, we also study a model of total aortic stenosis that carries a mutation in protein kinase d2 (prkd2) (Giardoglu et al., 2021). prkd2 mutants initiate blood circulation but fail to maintain it, allowing the study of valve development in the absence of proper intracardiac blood flow pattern, a phenotype that would be instantly lethal in mammalian systems (Figure 2). Simultaneously, in a collaborative study with research groups at the University of Crete, we showed that early developmental exposure to diverse temperatures could be associated with a different cardiac morphology. Increasing the temperature during development caused ventricular roundness and decreased exercise performance (Dimitriadi et al., 2018) of adult fish. Our collaborators had already shown (Georda et al., 2010) that early, and relatively limited periods of exposure to different temperatures during development could affect
the body shape and several morphological characteristics, including caudal fin segmentation pattern (Christou et al., 2018) that are also relevant to aquaculture. Recently, they also set up a micro-CT scanning-based protocol and 3D morphometrics to quantify in detail the changes in heart morphology (Dimitriadi et al., 2021).

**Zebrfish models of Neurobiology**

In the early days of zebrafish research in Greece, there was also interest and work that focused on adult neurogenesis. In a series of studies, it was shown that there is sexual dimorphism in cerebellum cell proliferation (Ampatzis and Dermon 2007). BrdU incorporation in specific brain areas, as well as immunohistochemistry of several receptors in distinct brain regions enabled researchers to unravel the specific distribution of beta(2)-adrenergic receptors and subsequently to correlate this regional distribution to swimming behavior, anxiety-like behavior, novel tank exploration and habituation of adult zebrafish, as well as sexual dimorphisms (Ampatzis et al., 2008; Ampatzis and Dermon 2010; Ampatzis et al., 2012; Ampatzis and Dermon 2016).

A significant contribution of Greek zebrafish research in zebrafish behavior and husbandry was the introduction of a very sensitive protocol for measuring a vital stress marker (cortisol measurement) (Pavlidis et al., 2013; Samaras and Pavlidis 2020). The group went on and determined the underlying molecular mechanisms to the stress response, including cf (corticotropin-releasing factor) and bdnf (brain-derived neurotrophic factor) transcriptional dynamics (Pavlidis et al., 2015). These analyses, together with morphological malformations that were caused in a model of exercise-induced lordosis in juvenile zebrafish (Printzi et al., 2021) also brought zebrafish research closer to aquaculture species physiology. Research groups at the University of Crete, together with colleagues at the Hellenic Centre for Marine Research studied the physiology of infections from a relevant to aquaculture bacterial family: *Edwardsiella*. Such outbreaks account for severe biomass losses and financial damage (Katharios et al., 2019), making zebrafish research more relevant for aquaculture, an area that is important for the Greek economy.

**Zebrfish models of Regeneration**

It is increasingly becoming evident that the reactivation of the developmental pathways is necessary to drive regeneration. Although adult mammalian hearts have limited regenerative capacity, newborn mice are able to regenerate. As a result, the hypothesis that re-activating or suppressing specific signaling pathways after injury could stimulate the endogenous regenerating potential in mammals remains thought-provoking. My group studied cardiac valve regeneration in zebrafish, using an inducible tissue-specific genetic ablation model. We expresses in a valve-specific pattern, the Nitroreductase gene (UAS:Nitroreductase) and by adding metronidazole at various time points we induced valvular damage. We have established that zebrafish cardiac valves can regenerate and isolated regenerating hearts to identify genes that are differentially regulated upon valvular damage using RNASeq. We have characterized Notch and TGFβ signaling as indispensable during valve regeneration (Kefalos et al., 2019; Benso-Brito et al., 2020).

Zebrafish has also proven to be an excellent model because of its amazing regenerative potential to functionally heal a spinal cord injury (SCI). In collaboration with several groups in Germany, we have analysed how perivascular cells and the specific ECM components they secrete, promote regeneration zebrafish, while similar cells create a non-permissive environment that leads to scar formation in mammals following SCI (Tsata et al., 2021).
Zebrafish in Translational Research and Precision Medicine

The high degree of genetic homology between zebrafish and Humans (it is estimated that more than 70% of Human Disease-causing genes have a clear orthologue in zebrafish), has led to the development of several zebrafish Human Disease Models. Furthermore, the ability to do high-throughput, phenotypic-driven screens allows the identification of potentially valuable preclinical molecules that could lead to drug development. A paradigm study for this approach is the generation of a zebrafish transgenic line carrying the 2057del2 mutation in the plakoglobin gene (Asimaki et al., 2014) that causes the Naxos disease (Protonotarios et al., 1986). Following the characterization of the phenotype, the authors conducted a high-throughput screen and identified a suppressor of cardiac mortality in fish. Then they continued and verified these results in rat cardiomyocytes carrying the plakoglobin mutation (Asimaki et al., 2014).

My group was able to study a Human adult-onset peripheral neuropathy: Charcot-Marie-Tooth (CMT), CMT2D caused by mutations in the Glycyl t-RNA synthetase (GARS). We identified and characterized a mutant line in the zebrafish gars where a Threonine to Lysine mutation in the dimerization domain inhibited protein dimerization and function. The embryos showed progressive loss of neuromuscular junctions (Malissovas et al., 2016). Using Human characterized pathogenic alleles (all were in conserved amino acids), we were able to show that dimerization between a mutant monomer and the wild-type is required for the autosomal dominant disease and indicate that a dominant-negative effect may be a potential mechanism of GARS-mediated CMT disease. It also provided us with a model where new variants of unknown significance in the GARS gene, could be tested in vivo, and could look for suppressors of the phenotype as well. This approach is being currently exploited by several Human Genetics groups studying variants of unknown function or novel genes in Human Development. For example, mutations in DYNC1I2 (Dynein Cytoplasmic 1 Intermediate Chain 2), encoding a component of the cytoplasmic dynein 1 complex was identified as a putative candidate gene first in a Pakistani family with microcephaly and intellectual disabilities and was shown in zebrafish to cause reduced head size (Ansar et al., 2019). In addition, mutations in the DDX3X (DEAD-Box Helicase 3 X-Linked) were identified that putatively cause intellectual disabilities in two male siblings. DDX3X is necessary for canonical Wnt signaling and therefore there is a functional assay to evaluate DDX3X variants. It is based on their ability, when coinjected with Wnt, to interact and cause ventralization of zebrafish embryos. This observation allowed the functional verification and characterization of a novel variant of unknown significance as a hypomorph, providing in vivo evidence for this rare genetic disease (Kellaris et al., 2018).

Besides Human Genetic analyses using zebrafish, my group has been also involved in several medium to high-throughput phenotypic screens to identify novel Bioactive compounds derived from various sources, including Natural Compound extracts. We have shown that crocins can regulate sugar levels in vivo using whole zebrafish embryos sugar measurements and the pancreas-specific transgenic line Tg(ins::DsRed) (Kakkouri et al., 2020). Mastic essential oil, a highly valued product unique to Greece, has also been studied in vivo using zebrafish. A group at the University of Ioannina analyzed the toxicological profile and immune response transcriptomic profile following addition of mastic essential oil in zebrafish embryo water, as well as its developmental effect on lateral line differentiation (Serifi et al.,...
My lab has been systematically screening for inhibitors of melanocyte differentiation. During this process that initially cannot differentiate between a melanin synthesis inhibitor or a melanocyte differentiation inhibitor, we identified and characterized two natural extracts from *Morus alba* and *Crataegus pycnoloba*. We showed that both work through melanin synthesis inhibition with potential usage in cosmetics, or as food supplements in aquaculture (Chaita et al., 2017; Agalou et al., 2018). We continue searching for melanocyte differentiation inhibitors with a potential application to treat metastatic melanoma.

There have been numerous zebrafish cancer models developed during the last 10 years. The high degree of homology between humans and zebrafish allows the creation of transgenic zebrafish lines introducing human oncogenes under a tissue-specific promoter. Zebrafish cancer models and especially the emerging powerful models of cancer cell xenotransplantations can also be used to screen for cancer biosensors. We have tested such a molecule *in vivo* that reacts with biothiols in cancer cells, emits near-infrared fluorescence and therefore could be used as a non-invasive cancer probe (Diamantis et al., 2021). Currently, these are well-established protocols in several labs and the metastatic behavior of cancer lines as well as their angiogenic activity and immune-triggering responses can be followed *in vivo*. There is an excellent correlation between the zebrafish results with mammalian models and their *in vivo* response to treatment with existing chemotherapies. The main advantage of zebrafish is the non-invasive *in vivo* imaging of cancer progression as well as the limited time needed to monitor these responses, which in most cases ranges from 4–10 days compared to several weeks in mouse models. Zebrafish patient-derived xenografts (zPDX) could distinguish functional tumor behaviors in just 4 days and showed differential sensitivity to colorectal cancer therapy (Fior et al., 2017). During the last year and using funding from the OpenScreenGR infrastructure grant where the Beis lab participates, we established the xenotransplantation protocol. We have successfully injected and tested the metastatic potential of several human cancer cell lines, including lung cancer and melanoma. We labeled cancer cells with the lipophilic dye DI and injected 200–10,000 cells depending on the line. In addition, we did a pilot screen using the lung cancer cell line (A549) and the reference drug gemcitabine, showing inhibition of the engraftment, proliferation and metastasis of cancer cells following xenotransplantation *in vivo* (Figure 3). It will be a great benefit for the Greek research community to establish a protocol for zebrafish patient-derived xenografts (also known as zebrafish avatars) (Costa et al., 2020) that could allow the patient-specific optimization of cancer therapy.

**Zebrafish in toxicology**

In the phenotype-driven screens a critical component for the characterization of extracts is to estimate their toxicity, in order to unravel and understand the actual underlying phenotype. There is an OECD-approved fish embryo lethality test that is used to determine lethal concentration (LC) parameters such as LC50 (OECD 236). Several Greek labs have used the zebrafish model to study the toxicological properties of various compounds. My group has characterized the *in vivo* effects during the development of the emerging pollutants, Benzotriazoles (Damalas et al., 2018).

Several other groups have used zebrafish embryo screens, as a toxicology model. At the University of Crete, they studied the toxicological properties and effects of waterborne Cadmium on adult fish unraveling an unexpected non-linear response to Cd (Renieri et al., 2017) and at the Benaki Phytopathological Institute in Athens, they
are integrating the zebrafish embryo test in their standard developmental toxicology analyses. They have shown that triadimefon (a widely used triazole fungicide) causes developmental defects (Zoupa and Machera 2017). The zebrafish embryo test can also be easily used to study the additive effects of different chemicals used in mixtures. This can be tested in a series of doses to check for morphological defects or lethality as an end-point (Zoupa et al., 2020).

Groups at the University of Ioannina, tested the cardiotoxic effects of the anticancer drug adriamycin (Orfanidou et al., 2013) and evaluated the toxicity of two molecules with anticancer properties N-(p-coumaroyl) serotonin (CS) (Lazari et al., 2017) and deglucohellebrin (DGH) which is extracted from the roots of Helleborus (Vartholomatos et al., 2020). They also studied the effects of cannabidol (a degradation product of the cannabis metabolite Δ9-tetrahydrocannabinol) during development as well as in survival and the metabolism of zebrafish embryos (Chousidis et al., 2020a).

A challenge with novel compounds used in industry is the evaluation of their toxicity. The impact of carbon nanodots (CNDs) and Carbon quantum dots (CQDs) was estimated using the metabolic profile and locomotor behavior of zebrafish embryos (Chousidis et al., 2020b; Chatzimitakos et al., 2021). In parallel, they also analyzed the transcriptional and behavioral impact on zebrafish embryos from a potent toxin produced by cyanobacteria: microcystin-LR (MCLR) (Papadimitriou et al., 2012; Tzima et al., 2017). A study with potential implications to the local environmental planning and monitoring of the Ioannina lake.

In a collaborative effort between laboratories at the Aristotle University of Thessaloniki and the University of Crete, researchers also utilized zebrafish as part of their toxicology analyses. They characterized at high resolution the physiological response of several species to either Titanium dioxide nanoparticles (TiO2-NPs) (Bobori et al., 2020) or magnetite nanoparticles (Kaloyianni et al., 2020). They were able to find specific biomarkers for each pollutant that were shared in several species. These biomarkers could be used to systematically monitor water pollution.

**Zebrafish as an *in vivo* model for Biochemistry and Cell Biology**

From the early days of zebrafish research, it was becoming obvious that it will be a powerful model for Cell Biology and Biochemistry (Beis and Stainier, 2006). The advances in BiolImaging and the transgenic lines that were generated over the years have provided all the necessary tools to exploit this potential to the fullest. Soon after the establishment of my lab in BRFAA we were able to follow the phenotype of a G-protein coupled receptor regulating protein. Knockdown of regulator of G protein signaling 4 (RGS4), a G i/o GTPase-activating protein (GAP), allowed us to show its involvement in β-cell migration and islet aggregation during the development of pancreas (Serafimidis et al., 2011).

The generation of zebrafish transgenic lines with artificial promoters allowed the imaging at cellular resolution of the activation of important signaling pathways. We participated in the developmental dissection of a widely used wnt signaling reporter line Tg(7xTCFXla.Siam:nlsmCherry) (Moro et al., 2012). We have also utilized the Notch reporter lines Tg(Tp1:venus-PEST) and the Tg(Tp1:h2B:mCherry) in several experiments, including valve regeneration (Kefalos et al., 2019). These lines show different kinetics of activation and signal maintenance due to the different fluorescent proteins they express. The destabilized venus-PEST is more dynamic, while mCherry can be used to visualize activation during longer periods. We also used the Hedgehog reporter line Tg(12xGliBS:mCherry-NLS), together with researchers at the University of Ioannina to show that the oncoprotein (protein phosphatase 2A inhibitor 2)
SET/I2PP2A inhibits Gli1 transcription (Serifi et al., 2021). Our collaborators at Ioannina, have used zebrafish to functionally verify *in vivo* and support Biochemical data regarding several signaling pathways. They studied the effect of Prothymosin alpha (*ProTα*) in cell proliferation and apoptosis during zebrafish embryonic development (Emmanouilidou et al., 2013). They also studied the role of the oncoprotein SET/I2PP2A during development of the zebrafish sensory system. They showed differential expression pattern following knockdown of the *set* genes in several organs, including the eye retina, the ear and the lateral line (Serifi et al., 2016).

**Outlook for zebrafish research in Greece**

The International or European Zebrafish meetings are the most diverse meetings I attend, reflecting the plethora of applications one can utilize zebrafish in research. I attempted to highlight some of these. There are now several labs working on zebrafish and considering the ever-increasing number of requests I get for new collaborations, even more research groups are interested in embarking on zebrafish-based research. Once funding in Greece, and especially for Infrastructures, becomes regular it will be crucial to establish a Greek Zebrafish Resource Center, with an open access policy to the Greek research community. This would be an essential addition to Greek Research Infrastructure for several reasons: a centralized zebrafish facility will be cost-effective, efficient and it will help Greek research groups in numerous and very versatile projects. A centralized screening facility for Bioactive compounds, that could boost zebrafish research and preclinical drug development programs in Greece. It would also be an ideal place to educate a new generation of Developmental Biologists in Greece. Zebrafish is particularly suited for hands-on laboratory research. Considering the versatility of small, easy to set-up experiments for undergraduates, it should also be integrated in the curriculum of every Biology or Medical department.

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Figure 1: Adult zebrafish swimming in the BRFAA aquarium.
Fig. 2. *prkd2* mutants exhibit a complete outflow tract stenosis. Confocal image of an embryonic heart from *prkd2* (*protein kinase D2*) mutant zebrafish depicting the cardiac endothelium with *Tg(kdrl::eGFP)* and circulating blood cells with *Tg(gata1::DsRed)*. Panels are pseudocolored, inspired by Andy Warhol’s art movement. *prkd2* embryos exhibit the extreme phenotype of outflow stenosis (arrow) and total retrograde blood flow from the ventricle to the atrium and no blood circulation by 72 hours post fertilization (hpf). They however survive, with a beating heart, for up to 120 hpf.
Fig. 3. Gemcitabine inhibits the growth of A549 cancer cells in vivo. We labeled the lung cancer cell line (A549) with the lipophilic dye DI and injected ~200 cells per embryo at 2 days post fertilization (dpf). Using the reference drug gemcitabine at 10μM, we showed inhibition of the engraftment, proliferation and metastasis of cancer cells in vivo within 5 days post injection (dpi). This approach can be used for any cancer cell line or zebrafish Patient-Derived Xenografts (zPDX).