FONDAZIONE GUIDO BERNARDINI
BETTER EDUCATION FOR BETTER SCIENCE

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

AISAL

ZFIM 2022

3RD ITALIAN ZEBRAFISH MEETING

NAPLES, 9,10,11 FEBRUARY 2022

PROCEEDINGS

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ZFIM 2022

3RD ITALIAN ZEBRAFISH MEETING

Oral Presentations

Oral Presentations

Title

Cell dynamics underlying the 3D organization of the zebrafish statoacoustic ganglion

Presenting Author

Aitor Bañón, PhD Student

Authors

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Content

Cranial ganglia are important neuronal structures in charge of transmitting sensory information to the central nervous system. One of the most complex ganglions is the statoacoustic ganglion (SAG). The SAG locates ventromedial to the inner ear and connects the hair cells of the inner ear with the corresponding neurons of the brainstem. SAG neurons derive from neuronal precursors that are born inside the inner ear. During development, the neuronal precursors delaminate outside of the otic epithelium, migrate to various positions and finally coalesce into lobes with distinct orientation. Depending on the neuron position, it will innervate the different vestibular or auditory hair cells. It remains unclear how delaminated neurons migrate, position correctly and finally establish proper innervation patterns resulting into the SAG final shape and organization. In order to address these questions, we have developed strategies to analyze cell behavior of otic neurons at single-cell level by photoconversion and photoablation experiments in reporter lines, and by disruption of candidate molecules using both Gal-UAS and CRISPR systems (Cas13 and Cas9). Imaging and tracking of otic neuroblasts show that cells migrate non-collectively and actively, producing membrane protrusions and filopodia. Delaminated neuroblasts migrate and coalesce in a stereotypical manner towards a nucleating region, causing the expansion of the anterior SAG lobe. Thanks to photoconversion experiments, we show that this nucleating region is populated by pioneer neuroblasts that also extend the pioneer axons. We observe that these pioneer axons act as scaffolds for delaminated neuroblasts to crawl and generate the posterior SAG lobe. Ablation of pioneer SAG neuroblasts causes defects in cell migration and axon crawling, indicating a relevant role of the pioneer cells in the organization and growth of the SAG. We are now addressing the role of two candidate molecules, Contactin2 (Cntn2) and Cxcl14, in neuronal coalescence and crawling. Cntn2 is a membrane bound protein that works via cell-cell homophilic interactions and is highly expressed in the SAG. Preliminary knock-down experiments of Cntn2 with CRISPR-Cas13 show SAG shape defects. Cxcl14 is a chemokine specifically expressed initially in the otic placode and later in hair cells. Preliminary results of downregulating Cxcl14 shows reduced pioneer SAG neuron migration, axon absence and altered crawling of SAG cells. Altogether, our work uncovers the molecular mechanisms by which cranial ganglia acquire its particular 3D shape and innervation patterns.

This work was supported by AEI-BFU2017-82723-P (FEDER) and by Maria de Maeztu Award CEX2018-000792-M from MCIN and AEI. Aitor Bañón is supported by FPU17/03287.

Oral Presentations

Title

Plasticity in retinal lineage decisions results in tissue robustness

Presenting Author

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Authors

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Content

During central nervous system development, multipotent progenitors need to commit to different neuronal fates in a controlled manner. This ensures that the correct neurons arise in the right numbers, proportions and with the correct timing. If this process is compromised, impairments in neuronal connectivity and organ hypoplasia can occur, with severe functional consequences. Therefore, understanding what fate decisions progenitors undergo over time, the degree of plasticity of these decisions and how this plasticity is achieved is of utmost importance to understand nervous system assembly.

Previous studies in the vertebrate retina demonstrated that retinal progenitors are multipotent. However, it is still not known whether and to what degree progenitors' competence – what neuronal types progenitors can generate at distinct developmental times – changes during development and whether different fates arise through deterministic or probabilistic processes.

To assess the journey from progenitor to neuron, I take advantage of the developing zebrafish retina that hosts 6 major neuronal types. The fast development and transparency of the zebrafish embryo allows us to follow progenitor's lineages with live imaging in vivo. By following neurogenic progenitors' lineages, I investigated the plasticity of progenitor's competence both in normal and in genetically perturbed conditions, when perturbing the emergence of single or multiple neuronal fates.

My studies revealed that within the same lineage, deterministic and probabilistic fate decisions can occur: progenitor's divisions generate always one photoreceptor and one sister cell, whose fate is acquired with different, time dependent probabilities.

When interfering with the probabilistic fate decision, we found that progenitors adapt their competence by generating later fates at earlier developmental times and vice versa, a process called fate switch. This fate switch at the lineage level results in robustness of the retinal tissue structure.

Combining experimental observations with statistical modeling allowed us to understand what interactions between key transcription factors underlie this adaptiveness in fate decisions.

We find that upon perturbation of the deterministic fate decision, the lineage cannot adapt through a simple fate switch mechanism and this leads to severe tissue defects.

Harnessing the imaging potential of the zebrafish embryo, we could investigate fate decisions processes in a nervous system at an unprecedented level, unraveling new rules of cell fate decision making.

Oral Presentations

Title

The stimulatory effect of the synthetic sulfolipid Sulfavant A on zebrafish microglia

Presenting Author

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Content

Introduction: Microglial cells represent the resident macrophages of the brain playing an important role in defence of neurons from different types of insults. In response to neuronal damage, microglia adopt an activated phenotype that typically includes a rounded morphology. In most neurodegenerative and neurological diseases, the activation of microglia is thought to play diverse roles. In this context, the manipulation of microglial activities by natural small molecules is a new and compelling therapeutic strategy. Zebrafish has many advantages for studying microglial response and functions in the living brain. Early zebrafish larvae have a fully functional microglia able to clear apoptotic neurons, to interact directly with highly active neurons and to respond to neuronal injuries. Here, we investigated the stimulatory properties of Sulfavant A, a synthetic analog of natural glycolipids, on the zebrafish microglia.

Results: Sulfavant A primes human cells of the immune system, as dendritic cells and microglia, by inducing maturation that involves a redistribution of major histocompatibility complex molecules to the dendritic cells surface, an increase in the surface expression of costimulatory molecules, morphological changes, phagocytosis, and antigen presentation. By using in vivo imaging and in situ hybridization technique, we analysed the behaviour of microglial cells in control and Sulfavant A pre-treated zebrafish larvae at 3 day-post fertilization to evaluate the promising immunomodulatory role of this lipid molecule. To stimulate microglia, zebrafish larvae were exposed to a pro-epileptic agent, the pentylenetetrazole, able to induce a generalized convulsive-like behaviour and to provoke inflammatory responses as well as oxidative stress. The analysis of area and density of microglia revealed a cell body reduction and an increase of branching of microglial cells in the brain of pentylenetetrazole treated zebrafish. In the brain of pentylenetetrazole exposed larvae, pre-treatment with Sulfavant A promoted the accumulation of microglial cells with an enlarged and amoeboid-like shape. Expression analysis of inflammatory genes revealed a more pronounced response of microglial cells when pre-conditioned with Sulfavant A. To evaluate the effect of Sulfavant A on microglial phagocytosis, we performed an in vivo assay using a pH-sensitive dye conjugated with zymosan particles, injected into the brain of control and Sulfavant A pre-treated zebrafish larvae. In one hour, we detected a faster ability of Sulfavant A pre-treated microglia to phagocyte zymosan particles compared to control.

Conclusions: Taken together, these results indicate that Sulfavant A act as immunomodulant molecule on microglial cells, improving microglia response and function. In the near future, we aim to identify the mechanism of action and the signalling pathway activated by Sulfavant A molecule in zebrafish. Our data further confirm zebrafish as a profitable model for the discovery of new natural product-derived molecules able to model microglial functions in therapeutic treatments for nervous system diseases.

Title

Zebrafish scarb2a knockout reveals a novel role for endo-lysosomal machinery during neurovascular development

Presenting Author

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Content

Neurovascular development is the parallel emergence and patterning of the central nervous system and the vascular system during embryogenesis. These complex events lie upon specific interactions between radial glia cells and endothelial cells. In this work, we describe a novel zebrafish mutant for scarb2a, a gene involved in lysosome biogenesis and maintenance whose deficiency is linked to Gaucher Disease, a member of the lysosomal storage disease family. scarb2a homozygous mutants present evident cerebrovascular defects with ectopic vascular sprouts and cranial hemorrhage at 3 days post fertilization. Nevertheless, our newly established scarb2a:GFP reporter revealed that its expression is detected in radial glial cells, but not in endothelial cells.
Moreover, we noticed that radial glial cells in the mutant present altered round morphology, over-proliferation and display defective migration. During central nervous system development, radial glial cells proliferate and differentiate into neurons and glial cells. Analysis of late developmental stages showed that mutants present reduced numbers of oligodendrocytes and increased amounts of neurons, suggesting the potential involvement of Scarb2 in neurogenesis. Mechanistically, we identified a strong decrease in Notch signaling in radial glial cells, concomitant with an altered endo-lysosomal patterning in the knock-out. Notch pathways is crucial for regulating radial glial cells proliferation and differentiation, and its activation requires intra-cellular trafficking mechanisms. Our results suggest a specific and still not fully understood role for Scarb2 and the endo-lysosomal machinery during central nervous system development. Moreover, scarb2a mutants represent a resourceful in vivo model to investigate the unexplored link between lysosomal storage diseases, cerebral angiogenesis, and neuronal impairment.

Keywords: zebrafish, neurovascular development, endo-lysosomal machinery, lysosomal storage diseases

Title

Neurovascular Interaction in Sensory Neuron Development

Presenting Author

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Content

Specialized sensory systems mediate the acquisition of the external world information. Of those, the inner ear is devoted in receiving and transmitting auditory and head movement inputs to the brain. Sensory neurons of the statoacoustic ganglion (SAG) connect hair cells to the second order neruons of the brainstem. Inner ear neuronal progenitors are first specified within the otic placode and subsequently neuroblasts delaminate out of the otic epithelium where they migrate, coalesce to finally differentiate into mature neurons. Little is known of how the sensory ganglion grows and matures and most singalling molecules identified play an intrinsic role within the SAG. In many organs, stem cell function depends on the communication with their niche partners.

Cranial sensory neurons develop in close proximity to blood vessels, however whether vasculature is an integral component of their niches is yet unknown. We have uncovered two separate, novel roles for vasculature in cranial sensory neurogenesis in zebrafish. The first involves precise spatiotemporal endothelial-neuroblast cytoneme contacts and Dll4-Notch signalling to restrain neuroblast proliferation. The second instead, requires blood flow to trigger

a transcriptional response that modifies neuroblast metabolic status and induces sensory neuron differentiation. In contrast, no role of sensory neurogenesis in vascular development is found, suggesting a unidirectional signalling from vasculature to sensory neuroblasts. Altogether, we demonstrate that the cranial vasculature constitutes a hitherto unrecognized niche component of the sensory ganglia that regulates the pace of their growth and differentiation dynamics. In conclusion, our results highlight for the first time the role of the neurovascular interaction in sensory neurogenesis (Taberner et al., Cell Reports 2020). The work was supported by AEIBFU2017-82723-P (FEDER) and by Maria de Maeztu Award CEX2018-000792-M from MCIN and AEI.

Oral Presentations

Title

MiR-204 is required for photoreceptor rod differentiation and maturation

Presenting Author

Giuliana Giamundo

Authors

Giuliana Giamundo, Daniela Intartaglia, Francesco Salierno, Ivan Conte.

Content

Timely generation of distinct neural cell types in appropriate numbers and morphology is fundamental for the generation of a functional retina. Rod photoreceptors are a specialized type of neuronal cell found in the retina that is capable of visual phototransduction. They are composed of an inner segment that contains the nucleus and the cilium, which extends into the Outer Segment (OS). The OS that contains visual pigment-bound membrane disks and the synaptic terminal that forms synaptic connections to the bipolar cells, relaying visual input. The OS is a light-sensitive cilium that captures photons thanks to membranous discs packed with photopigment protein Rhodopsin. They undergo through daily renewal process, by phagocytosis of outer segment fragments (POS) from retinal pigment epithelium (RPE), to refresh membrane and protein. Importantly, the mechanisms by which OS is initially formed from the photoreceptor cilium and continually renewed is not well understood and are of longstanding interest. Our study focuses on the role of miRNAs in controlling the molecular networks underlying the photoreceptor cell differentiation and maturation. MiRNAs
are non-coding RNAs that, generally, inhibit the expression of target genes and have been involved, among other processes, in both cell identity and morphology acquisition. In vertebrates, miR-204 is initially expressed in multipotent retina progenitors and then becomes restricted to differentiated retinal cells, including photoreceptor cells. How miR-204 expression in the retina is controlled and what are its precise functions are still unclear. We tackle some of these knowledge gaps. By taking advantage of rods specific miR-204 transgenic and miR-204 KO Medaka-fish lines, we are characterising the specific role of miR-204 in building and maintaining the light-sensitive OS of vertebrate rod during their differentiation and maturation, addressing one of the most fundamental unsolved problems in vision.

Oral Presentations

Title

Numerical discrimination by Archerfish

Presenting Author

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Content

KEYWORDS: numerical cognition, number discrimination, behavioural task, archerfish.

Non-symbolic numerical estimation is an important and well-studied cognitive ability that allows humans and other animals to interact successfully with their surroundings. The development of a “sense of number” is associated with fundamental biological needs that in many ecological contexts allow animals to estimate how many companions or enemies are around, or how much food is present in different patches.
Debates have arisen, however, as to whether non-human animals actually can learn abstract non-symbolic numerousness or whether they always rely on some continuous physical aspect of the stimuli, covarying with number. Since animals are dealing with sets of physical elements, numerical information is intrinsically melted with other non-numerical properties of the stimulus, such as the area, the density, the spatial frequency or the spatial arrangement of the items. Taking advantage of the fact that we recently developed a sophisticated script for the automatic generation of visual stimuli that can allow proper randomization and control of continuous physical variables in number sense experiments, here we investigated archerfish (Toxotes jaculatrix) non-symbolic numerical discrimination with accurate control for co-varying continuous physical stimulus attributes.
Archerfish are well-known for their particular hunting strategy, which consists of spitting at preys above the water surface with a precise jet of water thrown with the mouth. This attacking repertoire makes it very easy to train them to hit targets using operant conditioning.
Archerfish were trained to select one of two groups of black dots (Exp. 1: 3 vs. 6 elements; Exp. 2: 2 vs. 3 elements); these were controlled for several combinations of physical variables (elements’ size, overall area, overall perimeter, density and sparsity), ensuring that only numerical information was available. Training results showed that archerfish are capable of abstract numerical discrimination, not influenced by other continuous physical variables.
After reaching a learning criterion, archerfish were then tested with novel numerical comparison (2 vs. 3, 5 vs. 8 and 6 vs. 9 in Exp 1; 3 vs. 4, 3 vs. 6 in Exp 2) to check whether the rule they used in the training phase was based on a relative judgement (select the “largest” or “smallest” group) or on an absolute judgment (select a specific number of item). Results at test showed that fish generalize to novel numerical comparison according to a relative numerical rule rather than an absolute numerical rule. The spontaneous use of relative information of numerical groups may have ecological reasons, being more adaptive in a natural environment that constantly require numerical/quantity judgement.
In conclusion, our results provide clear evidence that under conditions of strict control of continuous physical variables archerfish can encode an abstract concept of number to support relative numerical judgement.

Oral Presentations

Title

Exploring the sense of number in zebrafish: a neurobiological approach

Presenting Author

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Content

Keywords: quantity discrimination, Approximate Number System, number cognition, immediate early genes

Abstract: The ability to deal with continuous (spatial or temporal) and discrete quantity (numerosity) developed from an evolutionarily conserved system for approximating non-symbolic numerical magnitude, which has been documented in a variety of species, including fish. In fish numerosity discrimination has been documented using spontaneous choice tests and operant conditioning procedures, however little is knowns about the neural correlates of this ability. By combining a habituation/dishabituation behavioral paradigm with molecular biology assays, we have recently identified part of the neural network associated with quantity discrimination in adult zebrafish brain. Zebrafish were habituated to groups of 3 or 9 small red dots for four consecutive days. During this phase, the dots changed in density, position and size (thus preventing the habituation of fish to a specific configuration of the stimulus though maintaining their numerousness and total surface area. During dishabituation, zebrafish faced a change (i) in number (from 3 to 9 dots or vice versa, with the same overall surface), or (ii) in shape (3 or 9 red squares instead of 3 or 9 dots with the same overall surface), or (iii) in size (with the same shape and number). A control group was tested with the same stimuli as during the habituation. Thirty minutes after the dishabituation test, zebrafish were sacrificed, their brains were collected and dissected to quantify the change in the expression levels of c-fos and egr-1 by quantitative polymerase chain reaction (qPCR) or probed with egr-1 in situ hybridization assays to identify the positional identity of neuronal correlates discriminating changes in quantity (number, size) or shape. Results showed an involvement of the retina and optic tectum in the encoding of continuous magnitude (e.g., a change in stimulus size). We also found a role of the habenula and the preglomerular complex, and of the caudal regions of the dorso-lateral and dorso-central pallium, in the encoding of discrete magnitude (e.g. change in numerosity). A response to shape discrimination was observed in the most rostral part of the dorso-central pallium. Results suggest an early involvement of thalamic and tectal areas for encoding of continuous quantity, and of more pallial (via talamac nuclei) regions for discrete quantity.

Title

Evolution of Adult Neurogenesis (ANG): a comparative study on Chondrichthyes as basal Vertebrates

Presenting Author

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Content

Adult Neurogenesis (ANG) is the phenomenon by which neurons are generated continuosly and integrated into the pre-existing circuits of an adult vertebrate brain. Its extent depends on the taxonomic group, but it is also a highly plastic process: it changes depending on the stage of ontogeny, the circadian rhythm and in response to physiological and pathological stimuli. For all these reasons, ANG can be considered an integrative biomarker of the organism's health and internal state and a potential biomarker of environmental stress.

The project is based on the investigation of ANG processes in cartilaginous fishes, starting by an initial descriptive analysis of the localization of neurogenic niches in five representative species of the main chondrichthyes clades: Chimaera monstruosa (Holocephali) for the basal clade, Torpedo marmorata and Raja asteria (Batoidea), Scyliohinus canicula (Selacimorpha-galeomorphii) and Somniosus microcephalus (Selacimorpha-squalomorphii) for Elasmobranchs.

Conventional neuroanatomical experimental approaches are used in this research. The Proliferating Cell Nuclear Antigen (PCNA) and phopshorylated Histone H3 (pH3), as a mitotic marker, and the glial marker S100β were combined in classical immuhistochemistry protocols on the abovementioned species: we found that Chondrichthyes possess two classes of stem cells with different anatomical localization. The cerebellum is characterized by pools of neuroepitelial cells in all species, as observed also in teleosts. The optic tecta of all species is characterized by active proliferation of radial glia, that appears to be specific of cartilaginous fishes.

Finally, the telencephalons of Scyliohinus canicula, Somniosus microcephalus and Chimaera monstruosa contains active proliferating radial glia and no astroglia, a situation typical of the embryonic mammalian brain, (putative neural Stem Cells), with a region specific distribution along the ventricular surfaces. In the telencephalon of Batoidea, we do not observe radial glia, but the peculiar presence of astroglia-like cells, suggesting a derived condition for these two species.

Title

Glucosyl-Sterols as novel signaling molecules in the nervous system: role in physiology and pathology

Presenting Author

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Content

Glucosyl-sterols are lipids composed of a sterol-derived molecule and a glucose moiety, but little is known about their function in cellular metabolism. However, alteration of their homeostasis has been recognized as one important risk factor for the onset of neurodegenerative diseases such as Parkinson's Disease and Amyotrophic Lateral Sclerosis, suggesting they play a fundamental role in the physiology of the nervous system and in the maintenance of neuronal functions.
The mode of action of glucosyl-sterols is still unknown but since they derive from sterols they may interact with both nuclear receptors and membrane receptors of neuronal cells, possibly competing with neurotransmitters and altering the proper response of neurons in signaling pathways. Moreover, it is tempting to speculate that they may also bind to specific receptors yet unknown.
Beta-glucosyl-sitosterol, a plant-derived glucosyl-sterol, was recognized as a contributor to the pathogenesis of the so-called Amyotrophic Lateral Sclerosis-Parkinsonism Dementia Complex, a pathology that shows Parkinson's Disease features, Amyotrophic Lateral Sclerosis features or both, thus representing an important neurodegeneration risk factor.
The aim of our research then consists in the study of the physiologic and pathologic roles of these molecules in neuronal cells and in the biology of the nervous system. To achieve this goal, we use the zebrafish as an animal model and to better understand the effects of glucosyl-sterols on phenotype, locomotor behaviour and gene expression we treat zebrafish larvae with beta-glucosyl-sitosterol.
Accumulation of this compound in the body of treated larvae was confirmed by mass spectrometry and the presence of aggregates in their intestine could be linked to an initial local inflammation that may then drive to a systemic inflammatory state. RT-qPCR with RNAs extracted from treated larvae indeed revealed an increasing trend in the expression of il1-beta, mmp9 and hmox, genes involved in inflammatory response and oxidative stress. This analysis also demonstrated a significative reduction in the expression of atg5 and lc3b, two main autophagy-related genes, suggesting a possible alteration of the autophagic pathway that can result in the occurrence of a proteopathy. These results seem to recapitulate the accumulation of intracellular aggregates typical of Parkinson's Disease, a process linked with autophagy impairment that contributes to cell death and neurodegeneration.
Administration of beta-glucosyl-sitosterol in mouse model determines the occurrence of ALS symptoms, such as the degeneration of motor neurons. A preliminary immunofluorescence experiment revealed an increase in caspase-3 positive puncta in the spinal cord of treated larvae as well, that may be linked with a higher level of apoptosis in this tissue.
We then exploited the Visual Motor Response assay to study the variations in the swimming pattern of treated larvae exposed to light and dark stimuli. We observed a general trend towards a decrease in the swimming performance of treated individuals, thus suggesting a lower propension to move or to properly respond to external stimuli.
To gain a wider view on the effects of Glucosyl-sterols on different metabolic pathways we aim to perform an RNA sequencing on individuals undergoing a chronic treatment with beta-glucosyl-sitosterol.

Keywords: Glucosyl-sterols, neurosteroids, neurodegenerative disease

Title

Ontogenetic changes of D-Asp in the brain of Nothobranchius furzeri

Presenting Author

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Content

D-aspartate (D-Asp) is the most abundant endogenous D-amino acid within mammalian brains during embryonic and early neonatal life. During adulthood, D-Asp levels decrease due to the concomitant onset of D-aspartate oxidase (DDO) activity, a flavoenzyme that selectively degrades dicarboxylic D-amino acids. Higher D-Asp levels in Ddo knockout mice brains trigger neuronal death, not seen in controls, suggesting that DDO may control the vulnerability to accelerated neurodegenerative processes. We conducted our research in a non-mammalian species, the African turquoise killifish, Nothobranchius furzeri, to test if the ontogenetic regulation of D-Asp and DDO in the brain is evolutionary conserved. This teleost fish is gaining new attention for aging research due to its short lifespan, during which it exhibits typical signs of aging. Previous studies have reported that DDO activity has been observed in the kidney and liver of some edible teleost fish, where D-aminoacids are metabolized following food intake, as well as in the brain, where its levels remain unchanged. Coherently with mammalian data, we show that D-Asp content in the brain of N. furzeri decreases dramatically after hatching, whereas D-serine levels remain unchanged. Unexpectedly, DDO mRNA levels do not increase over time similarly to the other two conserved genes involved in D-aminoacids metabolism, namely, D-amino acid oxidase (DAAO) and Serine racemase (SR). Interestingly, DDO, DAAO, and SR transcripts reveal a similar expression pattern along the neuroaxis. Furthermore, it is known in the murine brain that postnatal DDO gene expression is associated with progressive demethylation within its putative promoter region. Our preliminary data show comparable methylation levels within the putative DDO promoter region in N. furzeri, which is consistent with no significant changes in DDO mRNA expression. These findings shed light on the potential different regulation of D-aminoacids in the brains of mammalian and fish species, as well as the biological significance of the prominent postnatal DDO activity in vertebrate brains, which is still not yet clarified.

Title

Role of the BDNF in the zebrafish circadian system

Presenting Author

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Content

Brain-derived neurotrophic factor (BDNF) is one of the most important neurotrophin and plays a role in many pathways including neuronal growth, differentiation and plasticity, which is essential for learning and memory. We generated a zebrafish Bdnf null mutant line and characterized its circadian clock at molecular and behavioural level. We studied the expression pattern by qPCR of a set of clock and clock-related genes in null mutant and control larvae at 8 dpf under 12:12 light–dark (LD) cycles or constant darkness (DD). Larvae from both genotypes showed daily changes in the expression levels of all genes investigated. Differently, in DD condition control larvae maintained a rhythmic pattern of clock controlled-gene expression, whereas in bdnf-/- larvae the expression profiles of clock1a and clock2 became arrhythmic. Among the clock-controlled genes investigated the rhythmic transcription of aanat2, the enzyme that catalyses melatonin synthesis in the pineal gland, and of the melatonin receptor mtnr1aa were abolished in mutant larvae in DD conditions.
To test the presence of a working circadian timekeeping system, we recorded circadian locomotor activity in control and mutant larvae kept in DD for 3 days. In constant darkness condition bdnf+/+ larvae displayed a circadian locomotor activity. Conversely, bdnf-/- became immediately arrhythmic showing the absence of behavioural circadian rhythmicity. To confirm that the arrhythmic behavioural pattern was related to the lack of Bdnf, we performed a pharmacological rescue using 7,8-dihydroxyflavone hydrate (7,8-DHF) and showed a recovery of the circadian locomotor pattern in 7,8-DHF-treated bdnf-/- mutant larvae, compared to untreated knockout mutant larvae.
Here we demonstrated that Bdnf in zebrafish larvae is crucial for the generation of behavioural circadian rhythmicity. Furthermore, the generated zebrafish null mutant line might serve as a tool to investigate the role of Bdnf in different pathways including neuron development and synaptic transmission, visual light perception, regeneration and metabolism.

Keywords: Circadian clock; brain derived neurotrophic factor; CRISPR/Cas9

Oral Presentations

Title

A high-throughput system for Quality Control of Cas9 variants

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Content

The field of gene editing is rapidly moving towards the implementation of CRISPR/Cas9–based approaches to tackle human disease. The development of novel Cas9 variants is a crucial step to achieve precise and effective gene therapy.
To validate the safety of modified Cas9 versions, standardized experimental strategies for the evaluation of on-target endonuclease efficacy and potential toxicity, in vivo, are necessary since in vitro approaches are not predictive of the impact that such complex molecules can have on a living organism.
Here, we propose a zebrafish-based pipeline for quality control (QC) of two Cas9 protein variants, by evaluating, in a single assay, Cas9-induced toxicity, teratogenicity and on-target mutagenesis.
To perform this comparative analysis, we established a multistep approach composed by: 1) dose-range finding of Cas9/sgRNA complexes based on mortality evaluation at different timepoints after injection; 2) assessment of seven teratogenic phenotypes (heart edema, scoliosis, body deformity, craniofacial edema, defects in body axis curvature, eye diameter, pigmentation) to uncover putative developmental defects induced by the injection of Cas9/sgRNA complexes 3) high-throughput analysis of loss-of-function phenotypes by employing a fully automated microfluidic system 4) evaluation of Cas9 mutagenesis efficacy based on INDEL analysis of each analyzed larva for accurate correlation between phenotype and genotype. As proof of principle, we targeted the tyrosinase locus, since biallelic disruption of this gene results in loss of pigmentation, thus providing a direct readout of Cas9/sgRNA efficacy.
Overall, our results provide evidence supporting the use of zebrafish as a robust system to perform high throughput QC of new Cas9 protein variants.
This study paves the ground for the simultaneous validation of several gene therapy approaches employing innovative gene editing tools targeted to different therapeutic areas.

Oral Presentations

Title

Disease modeling by efficient genome editing using a near PAM-less base editor in vivo

Presenting Author

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Content

Base Editors are emerging as an innovative technology to introduce point mutations in complex genomes. So far, the requirement of an NGG Protospacer Adjacent Motif (PAM) at a suitable position often limits the editing possibility to model human pathological mutations in animals. Here we show that, using the CBE4max-SpRY variant recognizing the NRN PAM sequence, we can introduce point mutations for the first time in an animal model and achieved up to 100% efficiency, thus drastically increasing the base editing possibilities. With this near PAM-less base editor we can simultaneously mutate several genes and developed a co-selection method to identify the most edited embryos based on a simple visual screening. Finally, we applied our method to create a new zebrafish model for melanoma predisposition based on the simultaneous editing of multiple genes. Altogether, our results considerably expand the Base Editor application to introduce human disease-causing mutations in zebrafish.

Oral Presentations

Title

In vivo observation and modelling of the propagation of a neurotropic virus in zebrafish larvae and the role of type I interferons

Presenting Author

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Authors

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Content

The transparency of the zebrafish larva makes it an ideal system to study the dynamics of host-virus interactions. As a virus we used Sindbis Virus, a neurotropic alphavirus used o model viral encephalitis in mice, which can infect zebrafish efficiently. Using virus genome engineering, we could highlight virus-infected cells with several fluorescent reporters.

Using a combination of high-throughput and high-resolution imaging of transgenic reporter larvae, combined with in vivo cell depletion assays, we clarified the pathway of viral dissemination used by the virus, which relies on both long-distance axonal transport and short distance shedding. From the tail muscle, the virus uses Dorsal Root Ganglia (DRG) neurons as a gateway for the central nervous system. This results in an infection loop in the spinal cord with motoneurons and interneurons infected. Subsequently, the virus reaches the brain.

Through molecular biology and Interferons deficient larvae, we associated the viral waves of propagation to the host response and highlighted the role of type I interferons in controlling the infection.

Lastly, we used mathematical modelling for replicating the biological data obtained and infer different parameters like the number of virions produced by infected cells – which was surprisingly low-, the turnover ratio and the mode of action of interferons.

In conclusion, zebrafish larvae are a powerful model for virus-host interaction studies and in our work we managed to dissect the viral propagation pattern in a novel way, answering important biological question associated to virus movement from periphery to brain.

Title

TERRA is overexpressed in ALT brain tumors and induces the activation of the RNA sensing pathway

Presenting Author

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Authors

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Content

Keywords: RNA sensing pathway, cancer, telomere maintenance mechanisms, innate immunity

A large number of pediatric and 10% of adult brain tumors use *Alternative Lengthening of Telomeres* (ALT) to maintain telomere length and proliferation. A zebrafish model of brain cancer reproduces this mechanism (Idilli et al., 2020 a, b) and shows increased expression of *Telomeric Repeat-containing RNA* (TERRA) as the earliest event in ALT development. Here we investigated the potential of TERRA to activate the innate immune system detecting exogenous or pathological RNAs, as a mean to induce an inflammatory response that worsen progression and survival in pediatric brain cancer patients. First, we detected activation of *RIG-I Like Receptor* (RLR) pathway, with increased expression of sensors and *Interferon Response Genes* (IRGs) in conjunction with TERRA upregulation or overexpression. Second, we identified TERRA as an endogenous activator of the innate immune receptor, *Melanoma Differentiation-Associated protein 5* (MDA5), in tumors, and show that overexpression of MDA5 worsen inflammation and survival. By contrast, the overexpression of the card-less receptor, *Laboratory of Genetic and Physiology 2* (LGP2), in brain tumors, promotes survival and greatly reduces tumor size and inflammation in the juvenile zebrafish brain tumor model. Our results suggest that TERRA acts as a ligand for MDA5 in ALT brain tumors to increase inflammation, which worsen the progression of the disease. By contrast, overexpressing LGP2 in brain tumors has the opposite effect, probably by dampening the inflammatory response due to its inability to induce *Mitochondrial Antiviral Signalling* protein (MAVS) aggregation and further signaling. Thus, telomere maintenance through ALT mechanisms in brain cancer is accompanied by increased TERRA signaling through the RLR pathway, that promotes inflammation and counteracts anti-cancer immunity.

Title

The Interphotoreceptor matrix: investigating the role of IMPG2 in zebrafish retinal development and function

Presenting Author

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Content

Keywords: Retinitis pigmentosa, zebrafish, retina, photoreceptors, CRISPR/Cas9

Retinitis pigmentosa (RP) is one of the most commonly inherited retinal dystrophies, characterized by progressive degeneration of rod and cone photoreceptors. The genetic background of RP is heterogeneous, as are inheritance modes. Recent studies have reported that nonsense mutations in the interphotoreceptor matrix proteoglycan 2 (IMPG2) gene are associated with autosomal recessive RP in humans. This gene encodes the proteoglycan IMPG2, expressed in the interphotoreceptor matrix (IPM). IPM is the extracellular matrix that surrounds retinal photoreceptor outer segments and ellipsoids. IMPG2 is synthesized by rods and cones and it is secreted in the IPM. We chose zebrafish to investigate IMPG2 function and expression, as its retinal structure and organization is very similar to the human macula. In zebrafish, IMPG2 is present in two isoforms, Impg2a and Impg2b. Their expression as well as their role and possible differences are not yet known.

We analysed the evolutionary conservation of IMPG2, finding that it appears only in vertebrates. In many teleosts including zebrafish there are two paralogues, as foreseeable. Since IMPG2 protein structure has largely been unstudied, we then performed homology modelling of IMPG2 conserved domains both in human and in zebrafish, showing structure similarity of the domains in the two species. We finally investigated Impg2a and Impg2b mRNA and protein expression in zebrafish during early embryonic development and in the adult fish. RT-qPCR experiments revealed that Impg2a and Impg2b are transcribed from 3 days post fertilization (dpf), while in adults, both isoforms have an eye-specific expression. Western blots confirm a similar expression pattern for the proteins. Furthermore, immunohistochemistry experiments performed on retina sections showed that the expression of IMPG2 is found in the outer nuclear layer starting from 7 dpf and remains then present until adulthood.

Microinjection of antisense morpholino oligonucleotides (MOs), specific for each of the two isoforms provided preliminary evidence that IMPG2 is involved in eye development and RPE pigmentation in zebrafish. Moreover, morphant embryos show an increase in cell proliferation in the ciliary marginal zone. We are now characterizing the phenotype of Impg2a and Impg2b single and double mutant embryos, obtained by CRISPR/Cas9 technology. Our data points to the set-up of a new RP zebrafish model, that will allow us to discover disease mechanisms and pave the way for novel therapeutic approaches.

Title

Targeting Hedgehog and HDAC6 in acute myeloid leukemia in in vitro and zebrafish models

Presenting Author

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Content

In patients with Acute Myeloid Leukemia (AML) unknown drug-resistance mechanisms, driven by the abnormal activation of the Hedgehog (Hh) signaling, hamper the achievement of chemotherapy response. However, only one Hh inhibitor (glasdegib) is currently in use for AML treatment. Therefore, new therapeutic approaches are needed, and new molecular targets could be identified in the Hh signal cascade. The Hh pathway localizes on the membrane of the primary cilium, a microtubular structure derived from the centrosome and, therefore, expressed by non-proliferating mammalian cells. Since cancer cells, including AML cell lines, are characterized by a high rate of proliferation, they fail to present the primary cilium on their surface. Novel approaches to restore this structure on the surface of cancer cells are under investigation and most of them target the histone deacetylase HDAC6 as it controls cilium reabsorption. In our preliminary results, we verified that in AML patients' blood samples the expression levels of the Hh signaling are higher than healthy controls. Moreover, we demonstrated a positive correlation between Hh, HDAC6 and genes that confer tumor resistance (MDRs) both in patients and human AML cell lines. We confirmed the increased expression of hdac6 and MDRs also in a zebrafish model carrying the overexpression of the Hh pathway. In zebrafish we found that Hh hyperactivation generates a pre-leukemic phenotype, characterized by the hyperproliferation of the hematopoietic stem and progenitor cells (HSPCs). Interestingly, we rescued this hematopoietic phenotype with the HDAC6 inhibitor TubastatinA, but not with the Hh inhibitor cyclopamine. In line with this, the overexpression of the HDAC6 in zebrafish induced the hyperproliferation of HSPCs, confirming the role of HDAC6 hyperactivation in the pre-leukemic phenotype induction. To dissect the role of Hh/HDAC6 signaling in AML resistance and relapse we took advantage of zebrafish with Hh or HDAC6 overexpression or AML models and in vitro human AML cell lines in which we investigated the efficacy of a combination treatment with Hh and HDAC6 inhibitors and common chemotherapic agents.

Oral Presentations

Title

An in-vivo zebrafish model with cecr1b deficiency recapitulates human DADA2 phenotypes

Presenting Author

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Content

Deficiency of adenosine deaminase 2 (DADA2) is a rare and potentially lethal genetic disease caused by loss-of-function mutations in the ADA2 (CECR1) gene. Patients affected by DADA2 display neutropenia, bone marrow (BM) aplasia, immune dysregulation, systemic inflammation, vasculitis leading to vasculopathy and recurrent hemorrhagic strokes. Current therapies, mainly based on anti-TNF agents or allogeneic hematopoietic stem progenitor cell (HSPCs) transplantation, are not always resolutive. To date, advances in therapeutical treatments are hampered by the poor knowledge of the pathogenetic mechanisms and the lack of an animal model. Indeed, rodents do not have an ADA2 orthologue. Zebrafish (Danio rerio) harbors two ADA2 orthologues, cecr1a and cecr1b, with the latter sharing a conserved function with ADA2 and a specific expression during HSPCs differentiation. The cecr1b morpholino-mediated knock-down (KD) model showed neutropenia and intracranial hemorrhages. We characterized the hematopoietic and inflammatory phenotypes caused by cecr1b downregulation and investigated the mechanisms implicated in DADA2 pathogenesis. We successfully corrected the neutropenic phenotype of cecr1b-deficient embryos by administrating human granulocyte-colony stimulating factor (hG-CSF) or recombinant human ADA2 protein (rhADA2). Interestingly, we found that the neutropenic condition of cecr1b-KD embryos depends on an earlier defect in the HSPC population. Indeed, cecr1b-KD embryos showed a lower number of HSPCs in the caudal hematopoietic tissue (CHT), reminiscent of BM aplasia of severe DADA2 cases. Although HSPCs survival in the CHT was not affected, these cells exhibited a reduced proliferation capacity contributing to cytopenia. Moreover, we collected some preliminary data suggesting that the adenosine signaling pathway (adenosine2b receptor/cxcl8/runx) implicated in HSPC emergence might be altered in cecr1b-deficient embryos. Concerning the inflammatory phenotype, cecr1b-deficient embryos also displayed constitutive inflammation with enhanced expression of il-1beta, il-6 and tnf and polarization of macrophages towards the pro-inflammatory M1 population, as in DADA2 patients. In conclusion, our data provide, for the first time, the mechanistic link between DADA2 and hematological abnormalities and inflammation observed in patients and provided a valuable in-vivo model to develop new targeted therapies for this lethal disease.

Keywords: Adenosine deaminase 2, hematopoiesis, inflammation, neutropenia, immunity

Oral Presentations

Title

Dissecting mechanisms involved in the pathogenesis of Duchenne Muscular Dystrophy

Presenting Author

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Content

Duchenne muscular dystrophy (DMD) is an X-linked severe disorder characterized by progressive muscle wasting and weakness. Although therapies based on corticosteroids show positive effects on disease symptoms ameliorating the life expectancy of patients, an effective - or generally applicable - therapy is lacking. Thus, investigation of mechanisms dysregulated during DMD pathogenesis is necessary to find specific targets and to develop an effective therapy slowing down disease progression and ameliorating patients' lives.

Studies in animal models demonstrate that mitochondrial dysfunction due to dysregulation of Ca2+ homeostasis is a main cause of muscle fiber death in several types of muscular dystrophy. The long-lasting opening of the permeability transition pore (PTP), a high-conductance channel located in the inner mitochondrial membrane sensitive to high Ca2+ levels and modulated by matrix cyclophilin D, plays a major role in triggering muscle fiber death by favoring mitochondrial swelling and cristae remodeling with consequent release of the pro-apoptotic factor cytochrome c. We previously demonstrated that direct inhibition of PTP opening was able to prevent mitochondrial dysfunction and muscle degeneration in both DMD patient myoblasts and sapje myopathic zebrafish lacking dystrophin.

The aim of this work is to investigate mechanisms dysregulated during DMD progression, to identify the correct sequence of events that start with the absence of dystrophin and lead to Ca2+ dependent muscle fiber death.

We used sapje zebrafish mutant to identify: (i) which mechanisms link the absence of dystrophin to the effects of Ca2+ dysregulation; (ii) which signaling and metabolic pathways are altered during disease progression. To reach these goals we performed RNAseq analysis on sapje zebrafish mutants at different stages of disease progression and generated sapje mutants expressing fluorescent reporter genes under the control of specific gene promoter or pathway responsive elements to assess cell dynamics at the level of a single muscle fiber in a living organism.

RNAseq data from sapje zebrafish at 2 days post fertilization (dpf) indicated a significant increase of mitochondrial biogenesis and a strong impairment of muscle differentiation. At 5 dpf we observed an increase of oxidative stress and a stronger dysregulation of mitochondrial function and muscle differentiation. At 8 dpf we observed a strong activation of Voltage gated channels and impairment of numerous metabolic pathways. Further, preliminary analysis of signaling pathway dynamics in living sapje larvae at 2 dpf showed an upregulation of TGF beta and a downregulation of Notch and Shh both confirming defects in muscle differentiation and increasing of fiber death.

Sapje zebrafish mutant show phenotype features similar to that observed in DMD patients: (i) a dramatic disruption of muscle structure, (ii) mitochondrial dysfunction, (iii) a strong decrease of cell respiration, (iv) early death. The use of sapje zebrafish together with myoblasts from DMD patients will help to (i) fill the gap of knowledge about the mechanisms altered during DMD progression; (ii) identify new druggable targets to develop novel compounds to be used alone or in combination with PTP inhibitors, corticosteroids, genetic approaches. This may result in an effective treatment to slow down or prevent DMD progression.

Title

Morphofunctional characterization of a novel zebrafish polg2 mutant line modelling POLG-related disorders

Presenting Author

Raquel Brañas Casas, MSc, Department of Biology, University of Padua, Padua, Italy. Morphofunctional characterization of a novel zebrafish polg2 mutant line modelling POLG-related disorders

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Content

The mitochondrial DNA polymerase γ is an enzyme involved in mitochondrial DNA (mtDNA) replication and maintenance. The human holoenzyme is composed of a catalytic subunit (POLG) and two accessory subunits (POLG2). The POLG2 gene encodes for the accessory subunit of gamma polymerase (PolγB), which confers processivity to the POLG catalytic subunit (PolγA). In humans, the PolγB homodimer binds asymmetrically the catalytic subunit, strengthening the interaction with DNA and increasing the mtDNA replication. Mutations in POLG2 are also causative of POLG-related diseases in humans, leading to a subset of mitochondrial disorders, Mendelian inherited and characterized by mtDNA depletion or accumulation of multiple deletions,
presenting broad clinical expression and often leading to premature death in young age. To date, most patients identified with POLG2 related disorders were diagnosed with autosomal dominant progressive external ophthalmoplegia (adPEO) and variably displaying additional manifestations including skeletal muscle weakness, ataxia, depression and various neuro-sensory symptoms, typically worsening with the passing of the years. Therapies for these disorders are nowadays complex and non-curative, based on symptomatic therapy and supportive care, although clofilium tosylate (CLO) has been recently identified in our lab as a potential
therapeutic compound able to rescue POLG related phenotypes observed in a polg zebrafish mutant line.
To better understand the role of PolγB, we generated a stable zebrafish polg2 mutant line by CRISPR-Cas9 technology, carrying a 10-nucleotide deletion with frameshift mutation which leads to a premature stop codon (polg2ia303). In this work, we characterized the morphofunctional traits associated of polg2ia303 mutant line and conducted preliminary analysis to assess a potential efficacy of CLO treatment in rescuing some observed phenotypes. Our analysis revealed that polg2 homozygous mutants present slower development and decreased viability compared to wild type siblings, dying before entering juvenile stage. They phenocopy a key characteristic of human POLG-related disorders seen in human patients, that is a remarkable mtDNA depletion (MDD), as well as altered mitochondrial network and dynamics, and reduced mitochondrial respiration. Histological analyses were carried to assess whether polg2ia303 mutants present alterations among the high-energy demanding tissues, finding out a significant disorganization of skeletal muscle fibres. Consistent with the last finding, decreased motility of mutant larvae was highlighted by locomotor assays.
Since this mutation resulted in premature death during the larval stage, a new polg2 knock-in mutant line is currently being generated in our laboratory exploiting CRISPR/Cas9 genome editing.
Altogether, our results point at zebrafish as an effective model to study the etiopathology of human POLG-related disorders during early developmental phases, and a suitable platform for the screening of therapeutic drugs able to rescue Polg2-related defects.

Keywords: zebrafish, POLG, POLG2, mitochondria, mtDNA.

Title

Automated high-content drug screening in zebrafish xenografts identifies effective combination treatments against Ewing sarcoma

Presenting Author

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Content

Keywords:
High-content imaging, automated drug screening, larval xenotransplantation, Ewing sarcoma, anti-apoptotic protein inhibition

Abstract:
Xenotransplantation of human tumor cells into larval zebrafish holds great potential for the evaluation of novel compounds and therapeutic approaches for cancer treatment. Larval zebrafish xenografts offer live-imaging opportunities due to their transparency and furthermore cost-effective and fast drug screening at high-throughput. Due to the lack of an adequate genetic animal model of Ewing sarcoma, mouse xenotransplantations are currently the standard method to search for new effective compounds. However, mouse xenografts are expensive and time-consuming to establish and not well suited for high-throughput drug screening.
In this study, we transplant human Ewing sarcoma cells (sh-SKE17T) into 2 days post fertilization old zebrafish larvae and monitor them for several days. We show that not only human Ewing sarcoma cells can proliferate in our in vivo system, but we also provide a proof-of-principle that Ewing sarcoma xenografts can be applied for compound screening in a semi-automated high-throughput way. We screened a panel of 16 small compounds with reported in vitro efficacy against Ewing sarcoma cells. We found that two inhibitors of the anti-apoptotic proteins MCL-1 and BCL-XL, and especially their combination, show synergy in vitro and are highly effective against Ewing sarcoma in our xenograft model. We verified these results in xenografts with a second Ewing sarcoma cell line (A673-1c), and xenografts with patient-derived cells, confirming the potential of dual MCL-1/BCL-XL inhibition for Ewing sarcoma treatment. Furthermore, bioinformatic analysis of gene expression levels revealed MCL-1 and BCL-XL to be abundantly expressed across 93 Ewing sarcoma cell lines and tumor samples, suggesting that their combined inhibition could serve as a broadly applicable and potent strategy against Ewing sarcoma.

Title

Insight CLN8: Approaching therapies in the neuronal ceroid lipofuscinoses, using Zebrafish as a Tool

Presenting Author

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Content

Progressive myoclonus epilepsies (PMEs) are heterogeneous, as yet untreatable neurological conditions, whose main characterizing clinical features are myoclonus, drug-resistant epilepsy, and progressive neurodegeneration leading to dementia and early death. Based on their clinical features, autosomal recessively inherited PMEs include, among others, forms where myoclonus is in forefront of the clinical picture in lysosomal disorders, such as the neuronal ceroid lipofuscinoses (NCLs). NCLs are a group of lysosomal storage disorders, with onset at different ages, resulting from accumulation of neuronal and extra-neuronal autofluorescent storage material in lysosomes of many tissues and cell types. However, the pathological consequences of the disease are most prominent in the central nervous system and eyes. The storage material resembles ceroid and lipofuscin, that also accumulates during normal ageing. Lack of a therapy for most NCLs calls for the identification of new therapeutic molecules through the establishment of novel assays based on the associated molecular/biochemical disease pathways. To facilitate large screening of candidate molecules, a possible solution is to translate potential hits in relatively small models to estimate safety and efficacy. The CLN8 form have a late-infantile onset (after age 4 years), and is related to different pathomechanisms. In particular, CLN8 codes for a polytopic membrane protein, localized to the endoplasmic reticulum (ER) and shuttling between the ER and ER-Golgi intermediate complex, where it likely contributes to lipid homeostasis and lysosomal biogenesis.
Zebrafish (Danio rerio) is a suitable model to investigate a range of metabolic, neurological, and behavioural defects in clinical conditions associated with lysosomal storage, neurodegeneration, and epilepsy, such as the NCLs. It is also suited for large scale drug screening. To improve our understanding of sick and healthy CLN8 protein during neurodevelopment, we generated a new knock-out (KO) model in zebrafish, using CRISPR/cas9 system. The cln8-/- KO mutants recapitulate most of the pathophysiological features of PME and NCL. Indeed, behavioral analysis on cln8-/- KO homozygous mutants displayed impaired neuronal excitability and locomotor defects at different neurodevelopmental stages. Furthermore, a reduction in eye size, accumulation of SCMAS (subunit c of mitochondrial ATP synthase) and increased apoptosis in the brain were observed in our mutants compared to wild-type controls. Moreover, in the cln8-/- KO model we evaluated, with micro-oxygraphy at the single larva levels, a slight deficit of mitochondrial function compared to wild-type larvae.
Likewise, cln8-/- zebrafish showed differential expression of autophagy-related genes and an abnormal expression of the LC3 protein suggesting a dysregulated autophagic flux. Upon treatment with Trehalose, a naturally occurring disaccharide promoting autophagy, we observed rescue of the locomotor defects in mutant larvae.
Systematic analysis of EEG recordings coupled to calcium imaging in wild-type and mutant larvae treated with Trehalose is ongoing. The setting up of large-scale drug screening has just begun using FDA-approved small molecules modulating autophagy pathway. Overall, our data offer a new tool to prepare future pharmacological trials fighting the battle against NCL diseases.

Keywords: Neuronal Ceroid-Lipofuscinoses, CLN8, storage diseases, small molecule screening

Title

Signaling pathways dysregulation in zebrafish Foetal Growth Restriction models with cardiovascular changes: drug treatment implications

Presenting Author

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Content

Foetal Growth Restriction (FGR) refers to a pathological condition in which the foetus fails to grow to its biological potential because of chronic oxygen or nutrient deprivation. Nowadays, there is a lack of adequate intrauterine diagnostic approaches and therapeutic interventions in the paediatric period. Thus, dissecting the molecular and cellular mechanisms underlying FGR is a great challenge to improve diagnosis, prognosis and targeted treatment of this clinical entity to reduce the perinatal deaths. Zebrafish (Danio rerio) is a rapid, non-invasive, trustable and screenable model for the identification of key pathways and molecular factors involved in hypoxia-induced FGR, potentially targetable by pharmacological intervention. Our group benefited from the availability of transgenic-specific zebrafish lines for different signalling pathways relevant in embryonic growth. Taking advantage of these biosensors, the aim of our study is to investigate which signalling pathways are dysregulated by hypoxia-induced FGR conditions and can potentially act as either adverse or adaptive pathological responses in the developing embryo, paying specific attention to the zebrafish cardiovascular compartment. First, our study has demonstrated that hypoxia-treated zebrafish mimic a set of morphological and functional features detected in FGR cases, like growth retardation and delay in the nutrient uptake. The pericardial effusion and a disorganized vascular tree are two other features consistently detected in our models and associated with this pathology in human. Finally, our FGR zebrafish display impaired movements, another important aspect to consider since locomotor deficiencies have been described in FGR human infants. After confirming that the FGR phenotype in zebrafish mirrors the human one, we investigated if the FGR condition could induce changes in a set of signalling pathways associated with embryonic growth, using quantitative real-time PCR technique in human FGR umbilical cord samples and in situ hybridization technique in zebrafish embryos after hypoxic conditions. This analysis led to the identification of a panel of signals differently expressed in human and zebrafish FGR samples, in some cases with striking similarities between the two species. In particular, the two pathways that showed the same modification in human and zebrafish samples were Wnt/Beta-catenin and Jak/Stat3 signalling pathways, so we decided to chemically modulate them to understand how they are involved in FGR condition and if they can rescue the pathological phenotype. The pharmacological treatment of Jak/Stat3 signalling could not significantly modify the hypoxia-induced morphological phenotypes, suggesting a limited role of this pathway on body growth and pericardial homeostasis. However, the drug-induced regulation of Wnt/Beta-catenin pathway significantly affected body growth, pericardial size and vascular morphology under hypoxia, leading to an ameliorated or worsened phenotype in case of agonist or inhibitor treatment, respectively. This finding suggests that sustaining an already hyper-activated Wnt/Beta-catenin signalling in FGR may represent a rational intervention to support body growth and vascular development, while counteracting cardiac alterations. In conclusion, zebrafish proved to be an excellent model to understand the mechanisms behind the early signs of FGR impairments, owing to the strong similarities with human FGR phenotype and signalling dysregulation, and a suitable system for in vivo screening of pathway-targeted drugs.
Key words: Foetal Growth Restriction, hypoxia, zebrafish, signalling pathways, cardiovascular

Title

Zebrafish as a functional model to assess the effects of Extracellular Vesicles on innate immune response modulation

Presenting Author

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Content

Acute inflammation is a physiological response to tissue injury, infection and exposure to environmental stimuli. By its nature, the acute inflammatory response is self-limiting; persistent unresolved inflammation has long been recognized to play a fundamental role in the development of chronic diseases. How the human body respond to inflammatory stimuli and restore the homeostasis, is an open question in biomedical science. Extracellular vesicles (EVs) are a heterogeneous group of membrane vesicles, released from human cells under physiological and pathological conditions, that mediates cell-to-cell communication and immune response modulation. In particular, the cross-talk between EVs released from the human host and those released from the bacterial microbiota might influence the response to the inflammatory stimulus. We recently demonstrated that exposure to an environmental inflammatory stimuli, such as particulate matter (PM), alters the release of both host and bacterial EVs in human subjects. In pathological conditions, such as infant's bronchiolitis (after RSV infection), PM exposure is associated with the severity of symptoms as it influences the release of both plasmatic and bacterial EVs, potentially affecting the inflammatory response. To assess this, we set up a methodological approach to functionally evaluate the immunomodulatory potential of human and bacterial EVs using zebrafish. We microinjected locally or systemically the embryos with EVs suspension and evaluate the host immune response modulation. We monitored in real-time the progression of the immune response through the use of transgenic lines and by assessing the expression level of pro- and anti-inflammatory markers. This approach will help unveiling the link between one environmental stimulus that triggers inflammation and the immunomodulatory potential of specific EVs population.

KEYWORDS: inflammation, Extracellular vesicles, particulate matter

Title

Zebrafish as animal model for melanoma research: analysis of coding and non-coding BRAFV600E variants

Presenting Author

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Content

Melanoma is one of the deadliest skin cancers. While early stages are surgically treatable, later stages, characterized by infiltration and metastases, have an unfavorable prognosis. The most frequent mutation in malignant melanoma is the V600E substitution of the BRAF oncogene. This mutation constitutively activates MAPK pathway, promoting cell survival, proliferation and motility.
Among several pharmacological treatments developed for malignant melanoma, there are BRAF inhibitors (BRAFi), whose efficacy is high in the first months of treatment, but unfortunately is rapidly diminished by the insurgence of acquired resistance.

To identify new molecular factors involved in BRAFV600E-driven malignant transformation and response/resistance to BRAFi, we are developing melanoma models in zebrafish, by individually overexpressing BRAFV600E-ref and BRAFV600E-X1 splice variants. BRAFV600E-ref is the isoform universally used in melanoma modeling in zebrafish (reference). BRAFV600E-X1 is a poorly characterized isoform that, as we have discovered in our laboratory, always coexists with the ref.

Once injected at 1-cell stage with a plasmid expressing BRAFV600E-ref/X1 coding sequence only or BRAFV600E-ref/X1 coding sequence plus 3'UTR, adult p53-/- fish show altered pigmentation patterns and nevi, from which melanoma tumors originate. Interestingly, we have observed higher tumor incidence in fish expressing BRAFV600E-ref coding sequence vs BRAFV600E-X1 coding sequence, as well as in fish expressing each coding sequence vs coding sequence + 3'UTR. We have also observed that a delayed tumor onset correlates with a delayed appearance of nevi. At the moment, we are analyzing tumor samples collected so far, in order to identify histological similarities and differences between the two isoforms and to assess the influence of the respective regulatory sequences.

We have used the aforementioned BRAFV600E plasmids to generate stable transgenic lines as well. Such lines will allow us to test whether melanoma tumors originated in fish expressing BRAFV600E-ref coding sequence +/- 3'UTR vs BRAFV600E-X1 coding sequence +/- 3'UTR show a difference in terms of sensitivity to BRAFi and BRAFi-centered drug combinations.

Keywords:
melanoma, skin pigmentation, BRAFV600E-ref, BRAFV600E-X1, 3'UTR

Title

Relevance of ATM-dependent regulation of autophagy in Ataxia Telangiectasia

Presenting Author

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Content

Ataxia Telangiectasia is a rare childhood autosomal recessive disorder characterized by progressive neurodegeneration, immunodeficiency, cancer predisposition, gonadal atrophy, retarded growth, premature aging and hypersensitivity to ionizing radiation.

This pathology is caused by loss and gain of function mutations in the ATM gene, which encodes a 370 kDa serine/threonine protein kinase involved in many cellular pathways. Impairment of ATM-dependent DNA damage response has a well-established role in the pathogenesis of Ataxia Telangiectasia. Conversely, the significance of the ability of ATM to act as modulator of autophagy in the development of this disease is still largely obscure. Our aim is to investigate the role of ATM-dependent regulation of autophagy in Ataxia telangiectasia. In particular, we integrated the well-established in vitro model of Ataxia telangiectasia patients-derived lines with an in vivo approach based on zebrafish.

In a lymphoblastoid cell line derived from an Ataxia telangiectasia patient, the expression of Autophagy-Related 4C Cysteine Peptidase (ATG4C, mammalian autophagin-3) is reduced when compared to control cells. Interestingly, the reconstitution of wild type ATM expression in the same line is able to rescue ATG4C protein levels in Ataxia Telangiectasia cells. Using a specific inhibitor, we will further investigate if ATM-dependent regulation of ATG4C expression is mediated by ATM kinase activity. Moreover, a collection of small molecules with autophagy-inducing or autophagy-inhibiting activity will be employed to dissect the role of autophagy in the pathology of Ataxia Telangiectasia.

On the other hand, we are developing an in vivo zebrafish-based Ataxia Telangiectasia model to future testing of autophagy as possible therapeutic target for patients affected by this pathology. The atm gene is a single-copy gene in zebrafish, encoding for two different transcript isoforms (the longer atm-201 and the shorter atm-202). Protein sequence comparison showed around 50% of homology between zebrafish and ATM protein and in particular a conservation of phosphoinositide 3-kinase domain, which mediates ATM kinase activity. Performing real time qPCR, we demonstrated that atm is expressed during zebrafish embryonic development. In particular, atm mRNA has a maternal origin and zygotic expression displays a time-dependent increase during zebrafish embryo development, reaching its peak at 12 hours post-fertilization.

In addition, using CRISPR/Cas9 system, we generated loss-of-function mutations in atm gene in zebrafish. Gene-editing efficiency was confirmed by High Resolution Melting analysis on embryos injected ribonucleoprotein complex constituted by guideRNA and the endonuclease Cas9. Once reached the adult stage, the mosaic carriers of F0 generation transmitting atm mutations have been identified by outcross with WT fishes to obtain the heterozygous F1 generation.

In addition, zebrafish embryos were exposed to ATM inhibitor KU-55933 that was previously demonstrated to counteract the DNA damage-induced gamma-H2AX increase in zebrafish. Interestingly, larvae at 5 days post-fertilization treated with the higher doses of KU-55933 showed a hyperlocomotor behaviour.

Experiments aiming to study the effect of autophagy-inducing or autophagy-inhibiting activity are in progress, exploiting transgenic zebrafish embryos that allow to monitor the autophagic process in vivo.

Title

Mutations in the new disease-causing gene ARF3 have disruptive consequences on Golgi integrity and brain development

Presenting Author

Antonella Lauri, PhD

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Content

Keywords: Rare diseases, Golgi, GTPase, brain development

Rare diseases affect more than 400 million people worldwide. Most of these conditions involve nervous system development, maintenance and function and are often life-threatening already in children. Yet, despite the recent increase in disease-genes/variants discovery, many rare diseases remain undiagnosed and without targeted treatment due to poor knowledge of the underlying cellular and sub-cellular alterations. Our lab uses the zebrafish in vivo model system to validate candidate disease genes and genomic variants emerging from next generation sequencing of undiagnosed patients and to deepen our understanding of the mechanisms underlying newly discovered forms of RASopathies, tubulinopathies and golgipathies. Here we employed an functional genomics pipeline comprising human exome sequencing, in silico, in vitro and in vivo cell and developmental biology analysis in zebrafish to tackle a previously unidentified disease showing microcephaly, progressive cortical atrophy and skeletal anomalies. We identified de novo missense variants in ARF3, encoding a far neglected member of small GTPases of the RAS superfamily involved in Golgi-trafficking, as causative of the disease and provided first insights into ARF3 activity throughout vertebrate embryogenesis. In silico and biochemical investigations demonstrated that microcephaly-causing ARF3 mutations affect highly conserved residues regulating the catalytic activity of the protein participating in GTP binding. Experiments in live zebrafish embryos corroborated this finding and proved the disruptive consequences of aberrant ARF3 on trans-Golgi integrity. Comparable in vitro results substantiated the pathophysiological role the newly discovered ARF3 mutations, leading to Golgi fragmentation, as an underlying mechanism of this new form of Golgipathy. Our zebrafish models further validated the occurrence of a severe microcephalic trait, likewise the human condition and showed a fundamental perturbation of planar cell polarity (PCP)-dependent cell processes establishing the body plan axes, resembling a known effect caused by dominant ARF1. In conclusion, utilizing an integrated multi-level analysis (genomics, in silico, in vitro and in vivo), the work I will present 1. provides molecular classification for disease stratification, 2. offers a basic mechanistic knowledge of a previously unrecognized neurodevelopmental disorder and 3. document an obligate dependence on proper ARF3 function for organelles' homeostasis and early developmental processes.

Title

New insights into mitochondrial disorders: AKAP1 conditional knockout model in neuroendocrine system of zebrafish

Presenting Author

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Content

Mitochondrial dysfunction and decline in number are strongly linked to many pathologies. The structure, the function and the dynamic of the powerhouse of the cell are strictly regulated by several intracellular signaling pathways. The protein A-kinase anchoring protein 1 is considered a mitochondrial signaling hub. A-kinase anchoring protein 1 is a scaffold protein localized in the mitochondrial outer membrane important for driving mitochondrial respiration and mitochondrial DNA replication and quality control. A-kinase anchoring protein 1 deficiency triggers mitochondrial fragmentation and loss leading to decreased adenosine triphosphate production and enhanced mitochondrial reactive oxigen species-related apoptosis. Since the role that mitochondria dysfunction have in development and progression of neurological disease we focused our interest on the role of the A-kinase anchoring protein 1 in the neuroendocrine system aiming to dissect in vivo the molecular mechanisms of the protein activity in neurological and neurodegenerative diseases.
To investigate the role of A-kinase anchoring protein 1 in neuroendocrine system we generate an innovative zebrafish conditional, cell specific, knockout model. In the developed model the Cas9 protein (CRISPR/Cas9 technology) is expressed under the control of NeuroD1 promoter and targeted the deletion of the gene encoding for the A-kinase anchoring protein 1 in the neuroendocrine cells. Furthermore, NeuroD1 drive the expression of the green fluorescent protein in order to visualize and facilitate the study of the cell population involved.
The plasmid vector was injected in one cell-stage zebrafish embryo and positive eggs were screened for the presence of the green fluorescent protein and genotyped. Upon reaching sexual maturity, the positive zebrafish were mated to identify the founders. Interestingly, the parental generation showed a strong behavioral phenotype. The fishes loss the normal posture of the body and/or coordination of movements swimming on a side or showing a repetitive swimming in circular direction after different stimuli. This phenotype was observed also in the F1 generation. Moreover, modified zebrafish are characterized by morphological abnormalities including shorter body length and bent tail.
Future analyses will be perform to unraveling molecular mechanisms, however overall these data suggest that our model can be considered as a valid innovative model revealing a role for A-kinase anchoring protein 1 in neurological disease other than the already reported protective activity against neurodegeneration. The here presented model will allow the characterization of the altered molecular mechanisms associated to A-kinase anchoring protein 1 loss in neuroendocrine cells, potentially involving mitochondria, evidencing the potential of A-kinase anchoring protein 1 as target for therapy of various neurological disorder.

Title

Zebrafish larva as a powerful model to illuminate Legionella pneumophila infection in vivo

Presenting Author

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Content

Legionella pneumophila (L pneumophila) is a gram-negative bacterium commonly found in freshwater ecosystems, usually associated with protozoa. When aerosols containing L. pneumophila are inhaled by susceptible individuals, the bacteria reach the lungs where they can proliferate within alveolar macrophages, causing a severe type of pneumonia called legionellosis. L. pneumophila infection has been modelled in several animal and cell models, but these fail to reproduce important features of human disease. We established and characterized a zebrafish-L. pneumophila infection model to address the pathogenesis of the bacterium and the host immune response. We show that L. pneumophila is virulent in zebrafish larvae in a dose-dependent manner leading to larval death, although most immunocompetent larvae eliminate L. pneumophila. Using high-resolution confocal microscopy and transgenic zebrafish larvae, we show that macrophages represent the first line of defence, and that neutrophils cooperate to eliminate injected bacteria. In contrast, when macrophages or neutrophils are depleted, the "immunocompromised" larvae become highly susceptible to L. pneumophila and are unable to control and eliminate the bacteria, leading to a bacterial burden increase in the infected larvae concomitant with host death. We show that L. pneumophila injected into the bloodstream, invade and grow on the yolk sac region, a newfound feature in zebrafish larval infection models. On the yolk sac, L. pneumophila replicates forming large aggregates that cannot be cleared by macrophages and neutrophils, leading to death of the infected larvae. Strikingly, L. pneumophila virulence and yolk sac invasion is strictly dependent on the type 4 secretion system (T4SS), as the T4SS defective L. pneumophila mutant strain fails to establish sustained infection in zebrafish larvae regardless of the bacterial dose injected. The zebrafish model we established to study L. pneumophila infection, recapitulating key features of human infection, paves the way for new analysis and interrogation on how this bacterium modulates host cell functions, the virulence factors involved, and the crucial host factors counteracting the infection in vivo.

Key words: Legionella pneumophila, innate immune response, live imaging, neutrophils, macrophages.

Title

THE ROLE OF GR, MR AND STAT3 INTERPLAY IN THE REGULATION OF MITOCHONDRIAL METABOLISM

Presenting Author

Annachiara Tesoriere

Authors

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Content

The glucocorticoids receptor (GR) is a cytoplasmic protein that, after glucocorticoids binding, can translocate to nucleus and regulate the expression of target genes involved in cellular metabolism and stress response. Gr acts as a transcription factor, through direct interaction with gene promoter regions or through tethering with other transcription factors. It was already demonstrated that GR stimulates the expression of proteins involved in mitochondrial homeostasis and the recent detection of GR in mitochondria confirms a possible GR role in their metabolism. The goal of our research project is to identify how GR is involved in mitochondrial activities, evaluating not only both nuclear and mitochondrial GR functions, but also the role of GR tethering with other proteins. In details, we have focused on GR tethering with two important nuclear transcription factors: the mineralocorticoid receptor (MR), involved in the transcriptional response to glucocorticoids, and the Stat3 protein, a key factor of cellular metabolism.

We have started generating a gr zebrafish knock-out line and we identified new Gr nuclear targets involved in mitochondrial metabolism (ucp2, ucp3, slc25a25, slc25a43). Taking advantage of a zebrafish line with a point mutation (S357) in the DNA binding site of Gr protein – that allows to distinguish the Gr direct targets from the indirect ones - we revealed an important role of Gr tethering mechanisms in the regulation of the identified targets.

To evaluate a possible involvement of Mr in the expression of targets of interest, we have generated a mr zebrafish knock-out line. The results of our analysis seem to confirm a possible regulation by Gr-Mr tethering. In addition, both gr and mr knock-out lines showed alterations in mitochondrial calcium homeostasis and in vitamin D pathway, confirming that these two proteins are fundamental for mitochondrial homeostasis.

Our analysis of gr and mr knock-out lines transcriptome also highlighted a dysregulated expression of Stat3targets. Moreover, the literature reports all cited genes as Stat3 targets, suggesting that the identified mitochondrial proteins could be regulated by a Gr, Mr and Stat3 interplay. So, we have generated a stat3 knock-out zebrafish line and we are currently trying to confirm this hypothesis.

Interestingly both Stat3 and Gr have already been identified in mitochondria, but while the mitochondrial Stat3 functions are partially characterized, the Gr mitochondrial functions are still unknown. We have already demonstrated that mitochondrial Stat3 induces mitochondrial gene transcription, but this phenomenon is abrogated in gr zebrafish mutants. This result suggests that Gr-Stat3 interaction could exist also in mitochondria and could regulate DNA transcription. We are currently screening the potential Gr targets in mitochondrial DNA, trying to clarify the Gr role in mitochondrial transcriptional activity.

In addition, both gr and stat3 knock-out zebrafish larvae showed dysregulations in mitochondrial respiration (detected through seahorse assay) and in mitochondrial membrane potential (detected through TMRM staining). These results confirmed the hypothesis of a direct involvement of these proteins in mitochondrial metabolism, even if the Gr and Stat3 mechanisms of regulation of mitochondrial activities are still unknown and they are currently the objects of our investigations.

Title

Evaluation of innovative radiotherapy treatments in zebrafish.

Presenting Author

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Content

In the era of personalized therapy, radiotherapy is used in combination with compounds acting as radiation modifying agents to both improve the therapeutic index and obtain a more favorable compromise between tumor control and normal tissue toxicity. For this purpose, the zebrafish embryo represents an excellent model to use in the radiobiology field of scientific research for at least three main reasons: 1) embryogenesis is the most radiosensitive stage in the vertebrate life cycle, 2) the embryo undergo rapid cell division, and 3) the aqueous environment in which embryos develop favours homogeneity in the radiation dose distribution.

In order to implement innovative radiotherapy treatments, we describe the characterization of the biological effects induced in zebrafish embryos exposed to X-ray beams, alone or in combination with curcumin, a radiomodifier known for anti-oxidant and anti-tumor properties.

Distinct batches of 24 hpf embryos were exposed to X-rays at the clinical dose range of 0-15 Gy. For the combined treatment, embryos were pre-treated from 6 to 24 hpf with curcumin at concentrations of 0-10 µM, and subsequently irradiated using the abovementioned dose range. Sister batches of 6 hpf embryos were either used as untreated controls or subjected to single treatment with curcumin following the same experimental setting used in the combined treatment. Treated and control embryos were carefully examined by daily steromicroscope observation until 5 days post-fertilization, to estimate the mortality rate as well as developmental delay and alterations. In addition, behavioural analysis was performed to assess alteration in swimming capacity or delay in response to induced physical stimuli, as well as any possible variation in the heart rate values at 48 and 72 hpf.

We found that the single treatment with curcumin, at concentrations greater than 5 µM, and the X-ray single exposure in a dose-dependent manner, inflicted gross malformations (including pericardial and yolk-sac edema, skeleton defects, cardiac and vascular dysfunctions, and variation in the pigmentation degree), behavioural defects and lethality. In striking contrast, the occurrence of these phenotypic alterations was markedly reduced, at different extents, in embryos exposed to the combined treatment, strongly suggesting that the adverse effects induced by radiation were mitigated in these embryos by curcumin pre-treatment at non-lethal and non-toxic doses concentrations of 0-5 µM.

Additional experiments are planned to accomplish the characterization at a molecular level of the observed effects.

Keywords: radiation therapy, curcumin, embryogenesis.

Oral Presentations

Title

Intravitreal administration of rhNGF enhances regenerative processes in a zebrafish model of retinal degeneration

Presenting Author

Cocchiaro Pasquale

Authors

Pasquale Cocchiaro , Vincenzo Di Donato , Davide Rubbini, Rodolfo Mastropasqua , Marcello Allegretti , Flavio Mantelli , Andrea Aramini , Laura Brandolini

Content

Nerve growth factor (NGF) is the best characterized neurotrophin and it is known to play an important role in ocular homeostasis. Here, we demonstrated the expression of NGF receptors in adult zebrafish retina and optimized a light-induced retina degeneration (LID) zebrafish model that mimics human cone-rod disorders, demonstrating that intravitreal (IV) administration of rhNGF can boost zebrafish retinal regeneration in this model.
Adult zebrafish retinae exposed to 60 hours light irradiation (60 h LID) displayed evident reduction of outer nuclear layer (ONL) thickness and cell number with presence of apoptotic cells. Retinal histologic evaluation at different timepoints showed that IV therapeutic injection of rhNGF resulted in an increase of ONL thickness and cell number at late timepoints after damage (14 and 21 days post injury), ultimately accelerating retinal tissue recovery by driving retinal cell proliferation. At a molecular level, rhNGF activated the ERK1/2 pathway and enhanced the regenerative potential of Müller glia gfap- and vim- expressing cells by stimulating at early timepoints the expression of the photoreceptor regeneration factor Drgal1-L2.
Our results demonstrate the highly conserved nature of NGF canonical pathway in zebrafish and thus support the use of zebrafish models for testing new compounds with potential retinal regenerative properties. Moreover, the pro-regenerative effects of IV-injected NGF that we observed open the way to further studies aimed at evaluating its effects also in mammals, in order to expedite the development of novel rhNGF-based therapeutic approaches for ophthalmological disorders.

Title

Embryonic and larval exposure to propylparaben induces developmental and long-term neurotoxicity in the zebrafish model

Presenting Author

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Content

Propylparaben is one of the most used parabens in personal care products as a preservative, antimicrobial, and mold and yeast inhibitor. Based on early risk assessment conducted before 2000, propylparaben was considered safe for human use. However, later, research indicated parabens may modulate or disrupt the endocrine system and they were recently classified as endocrine disruptors. Most of the information on the harmful effects of propylparaben still relies on in vitro and in vivo experimental studies focused on reproductive toxicity. Moreover, these studies tend to analyze a range of high paraben concentrations, which may not represent realistic exposure concentrations, and do not consider sensitive endpoints as outcomes. The present study aims to investigate the propylparaben-related developmental and long-term neurotoxicity in the zebrafish model through an integrated approach and considering different endpoints. Zebrafish early-life stages were exposed for 4 days post-fertilization to two different concentrations of the chemical, an environmentally relevant concentration of human exposure (10 µg/L) and a toxicological concentration (1000 µg/L) and then raised to adult stage (180 days post-fertilization). The effects of propylparaben on brain development were investigated evaluating the expression of the neurodevelopment-related gene (cyp19a1b, pax6a, shank3a, gad1b), and cognitive-behavioral phenotype (open-field behavior, startle response, circadian rhythmicity, sociability, and brain functional asymmetries) in larval and adult specimens. Cyp19a1b mRNA increased in zebrafish larvae exposed to both concentrations of propylparaben, while no difference was observed for pax6a expression. The mRNA expression level of shank3a in treated larvae decreased, revealing a strong modulation of this gene during zebrafish development. Propylparaben treatment did also significantly change the mRNA level of gad1b in the 10 µg/L group, while only a downward trend was visible in the 1000 µg/L group. In adult zebrafish, cyp19a1b and pax6 genes showed a trend of increased expression at low concentration, while no differences were observed for high concentration. Gad1b expression was similar to the control; however, shank3a expression continued to be low, particularly at 1000 µg/L, and a similar trend was observed for 10 µg/L. Exposure to 1000 µg/L caused a reduction of thigmotaxis in zebrafish larvae and adults. Moreover, developmental exposure to 1000 µg/L and marginally to 10 µg/L caused a reduction of sociability in adult zebrafish. Activity, startle response to light, circadian rhythmicity, and functional brain asymmetry were not affected by propylparaben exposure. Overall, the results of the present study highlighted that embryonic exposure to low concentrations of propylparaben could be deleterious to the central nervous system development and elicit behavioral abnormalities in zebrafish at the developmental and adult stages. Zebrafish larvae are an alternative model that can be used to integrate the in vitro and rodent approaches in studying neurotoxicity of chemicals and the present research, using a multidisciplinary approach to characterize the acute and long-term toxicological effects of parabens on zebrafish neurodevelopment, could increase the value of zebrafish model in developmental neurotoxicity field.

Title

Caulerpin: a modulator of fish reproduction from a highly invasive species

Presenting Author

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Content

Biological invasions are among the main causes of biodiversity loss on a global scale, with negative ecological and economic consequences. The theme is of great concern for marine ecosystems and, in particular, for the Mediterranean Sea, which is considered a major hotspot of marine invasions. In this framework, the green macroalga Caulerpa cylindracea has come to the fore due to its high invasiveness in the Mediterranean. Actually, this alga forms impenetrable monocultures that negatively affect native seaweeds and seagrasses and the livelihoods of local fishermen. Moreover, the alga has become a favorite food of native fish, including the edible white sea bream Diplodus sargus. The novel alimentary habits were correlated with alterations in metabolic pathways, suggesting possible chemically-mediated detrimental effects of the new diet. However, the potential negative impact of specific bioactive metabolites from C. cylindracea on fish health remains still unclear. One of its components, the bisindolic alkaloid caulerpin, was seen to accumulate in fish livers correlating with higher transcriptional levels of vitellogenin. Here we show that the compound also accumulates in the gonads of D. sargus, further supporting its possible involvement in fish reproduction. In order to clarify the effects of caulerpin-based diet, we decided to employ the zebrafish model, adding purified caulerpin to its normal food. As results, we observed improved fish reproductive performance, significantly increasing fertility and embryos/larvae viability. These effects are in turn associated with caulerpin-mediated transcriptional induction of genes involved in the control of the hypothalamus-pituitary-gonadal axis and sexual hormones synthesis. Finally, morphological analysis of caulerpin-treated fish showed early ripening of the ovaries, associated with an increased gonado-somatic-index. In conclusion, our results seem to suggest that other algal metabolites (e.g. the toxic sesquiterpene caulerpenyne) could be responsible for the adverse effects of a Caulerpa-based diet on fish. Instead, the positive effects of caulerpin on fish reproduction and health pave the way for a possible future desirable valorization of C. cylindracea as a source of a bioactive compound able to improve fertility and reproduction of aquaculture fish species.

Title

Polyphenols show anti-inflammatory effects on intestinal inflammation in zebrafish (Danio rerio)

Presenting Author

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Content

The gastrointestinal system of aquatic animals, being almost in direct contact with the external environment and providing to the digestion and absorption of nutrients, represents an important barrier against infectious agents and toxins. In aquaculture, the diet administered may affect the gastrointestinal apparatus, inducing an inflammatory status with consequent negative effects on health and growth. The use of phytochemicals, such polyphenols, in animal farming seems to be a promising tool to preserve the health fish status. The aim of this study was to investigate the effect of chestnut shell (Castanea sativa) extract (CSE), rich in polyphenols (tannins), on intestinal inflammation zebrafish (Danio rerio) model. Intestinal inflammation was induced by the addition of 0.1% k-carrageenan to the diet for 3 days. To test the efficacy of CSE to counteract the inflammatory status, it was administered for seven days after K-carrageenan-induced inflammation. Morphological analysis showed how k-carrageenan led to gut lumen expansion, induced by thinning of intestinal folds, and increase of the goblet cell number. Moreover, an upregulation of pro-inflammatory (TNFα, COX2) factors and alteration in the number and ratio of taxonomic groups of bacteria, was detected. CSE ameliorate the inflammatory status, enhancing the growth of health helpful bacteria, such as Enterobacteriaceae and Pseudomonas, reducing the oxidative stress and inflammatory status (COX2) and activating the anti-inflammatory cytokines (Il-10). In conclusion, CSE acts as prebiotic on zebrafish gut microbiota validating the hypothesis that the use of extracts rich in polyphenols as food additives may ameliorate the intestinal inflammatory status and thus nutrition manipulation may be introduced as an easy to carry on practice in both aquaculture and medical clinic.

Title

Zebrafish, a successful tool for Aquaculture research

Presenting Author

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Content

Aquaculture is the breeding, rearing, and harvesting of fish, shellfish, algae, and other aquatic organisms. The rapid world population growth and the declining of world fish catches have created the need for additional protein sources for human nutrition. To this regard, the Food and Agriculture Organization of the United Nations and other relevant international Institutions (e.g., EU) have suggested aquaculture as strategic sector. The growth rate of the aquaculture sector is higher than any other food production industry with an estimated rate of 5.3 percent per year in the period 2001–2018. For supporting such as trend, there is a strong need of new research in many areas such as genetic, reproduction, nutrition, and welfare. Thanks to the small size, short generation time interval, prolificity, easy management and manipulation, zebrafish is an excellent tool for aquaculture research. In fact, the genetic similarity between zebrafish and other fish species of interest for the aquaculture sector is greater than that existing between zebrafish and mammals, such as human. Nonetheless, species-specific differences must be still considered. In the past decade, zebrafish proved to be a reliable model for in vivo studies on fish immune response, vaccines development, drugs screening, infectious and inflammatory diseases that are of some concern for the aquaculture industry. Furthermore, research has been carried out on fish nutrition, reproduction and behaviour contributing to improve fish growth performances, survival rate, husbandry procedures, farming stress management, disease resistance, among others. To replace fishmeal and improving aquaculture sustainability, studies on the use of plant (e.g., soybean meal, rapeseed meal, corn gluten meal, etc.) or animal (e.g., krill meal, poultry meal, yeast derived by-product, insect meal, etc.) alternative protein sources, have been also carried out.

At the Department of Veterinary Science of the University of Pisa, zebrafish has been used as fish model for studies concerning several aspects. To enhance immunity and fish health, 1,3-1,6 ß-glucan were tested on tissues regeneration (wound healing) and disease resistance (Edwardsiella tarda). Plant extracts known for their antioxidant or sedative properties (e.g., Cannabis sativa, chestnut, Melissa officinalis, etc.) were tested to limit the negative effects of stressful farming conditions (e.g., unbalanced diets, high fish stocking density, fish transportation, etc.). Dipeptides (carnosine and glycyl-proline) were also used to promote fish health and gut function. For improving aquaculture sustainability, bee pollen, Hermetia illucens meal, and yeast derived by-product, were used as feed ingredient for partially replacing fish feed or fishmeal in fish diet. Besides that, gingerol and 1,3-1,6 ß-glucan have been tested as possible food aid for patients affected by Muscular Dystrophy.

Considering that aquaculture is a relatively recent practice, there is still a relevant need of research for enhancing fish performances and health, and to cope with stress related to the farming practices (e.g., fish transportation and selections). In this context, zebrafish already represent a successful key tool for aquaculture research.

ZFIM 2022

3RD ITALIAN ZEBRAFISH MEETING

Poster Presentations

Poster Presentations

Title

Chromatin and gene expression dynamics during inner ear neurogenesis

Presenting Author

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Content

The inner ear is one of the most complex sensory organs of the head and is responsible for the senses of hearing and balance. Bipolar neurons of the inner ear are involved in transmitting sensory input captured by the hair cells to the brain. Otic neurogenesis starts with the specification of otic progenitors into neuronal precursors by the proneural gene neurog1 that activate another proneural gene, neuroD. NeuroD-positive neuroblasts then delaminate out of the epithelium and differentiate into neurons that extend axons. It has been shown in reprogramming studies that these proneural genes are pioneer factors, which means that they can target sequences of nucleosomal DNA and locally modify the epigenetic landscape into an active or repressed state. While these proneural genes are expressed in many cranial ganglia it remains unknown how, in addition to a pan-neuronal role, can induce specific neuronal identity transcriptional programs. In this work we explore how the chromatin landscape is rearranged during the process of otic neurogenesis and the transcription factors that might be terminal selectors and impinge otic neuronal identity.
In order to address this question in cells in their context, we have used the zebrafish reporter transgenic lines Tg(neurog1:dsRed), Tg(neurod:eGFP) and Tg(islet2b:eGFP) to purify by FACS the different temporal otic neuronal populations and analyze the chromatin state and gene expression by ATAC-seq and RNA-seq, respectively.
Preliminary analysis indicates that at early steps of neurogenesis most significant opened chromatin peaks correspond to transcription factor genes, while in postmitotic neurons the number of opened chromatin regions are less and mainly correspond to cell guidance and cytoskeleton associated genes. Regarding the opening of the chromatin, we detect regions opened prior to gene transcription, suggesting a pioneer factor role of neurog1. Most of these regions remain opened in all stages of neurogenesis but we identify few ones that close once cells become postmitotic. Some chromatin regions open specifically at later stages and correlate with the induction of gene transcription. We are currently analyzing the existence of a common transcription factor signature that could highlight us on the co-factors of the proneural factors in otic neurogenesis.

Keywords: transcriptomics, epigenetics, inner ear, otic neurogenesis, zebrafish.

Poster Presentations

Title

In vivo and 3D in vitro models: two different approches to study Malignant Pleural Mesothelioma

Presenting Author

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Content

Keywords
Zebrafish embryo, 3D scaffold, Mesothelioma

Background
Malignant pleural mesothelioma is a chemotherapy-resistant cancer linked to asbestos exposure. Despite recent advances in therapeutic approaches, patients affected by mesothelioma usually die within 1 year following diagnosis. Due to its low incidence (about 30,870 annual new cases), preclinical research lacks of innovative in vitro and in vivo models. We propose a 3D biomimetic collagen-based scaffold and zebrafish embryos models of malignant mesothelioma to help the understanding of the biology of this highly lethal cancer and to test new therapeutic approaches.

Methods
We used 4 cell lines to cover all the mesothelioma histotypes: NCI-H2452 (epithelioid), MSTO-211H (biphasic), CD60 and CD432 (sarcomatoid). Cancer cells were seeded on the 3D devices at different concentrations and through MTT assays were measured the 2D and 3D cultures viability. The scaffolds were also embedded in paraffin and stained with phalloidin for confocal imaging analysis to study the spatial distribution and morphological features. The effect of cisplatin and pemetrexed were tested in both culture types at the plasma peak concentrations.
Cells recovered from 2D and 3D cultures were injected in the perivitelline space of zebrafish Tg(fli1:eGFP)embryos at 2 dpf. The number of embryos with circulating and disseminated cells were monitored until 72 hpi.

Results
The optimal cell concentration resulted 100.000 cells per scaffold. The 3D cultures showed different morphologies depending by the histotypes. The epithelioid cells appeared with tapered and elongated shapes along the collagen fibers, while the bifasic cell line grown in cluster inside the scaffold porus. On the other hand, sarcomatoid cell lines acquired an ordinated and circular rearrangement covering all the porus area. The cell distribution resulted higher in the edge and lower in the scaffold core in all the histotypes.
The drugs sensitivity resulted not significantly different between the 2D and 3D cultures except for the epithelioid cell line treated with cisplatin.
The in vivo experiments showed a higher migration capability of NCI-H2452 and CD60 cells grown in 3D respect to the common monolayer devices while MSTO-211H displayed the opposite behavior.

Conclusion
Our work provides proof of concept of the ability of 3D cultures to maintain the original phenotype of the different mesothelioma hystotypes cells, and highlights the potential of the zebrafish model to provide a versatile in vivo system to study the tumor aggressivenes. Such models could be used in translational research studies for biomolecular analyses and drug screenings.

Title

The CRISPR toolbox in the elucidation of the role of sall1a during hair cell development

Presenting Author

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Content

Townes-Brocks syndrome is an autosomal dominant disorder characterized by mutations in the Spalt-like family transcription factor SALL1. Its clinical presentation involves the impairment of multiple organs. These include malformation of the limbs and ears, cardiomyopathy, renal dysfunction, imperforation of the anus and high-frequency sensorineural hearing loss. Most mutations described in this type of hearing loss have been linked to genes that are key in hair cell development. In Drosophila, the developmental role of sal in some organs has been previously described. Nevertheless, the role of sall1 in the vertebrate inner ear and how it might be causing hearing loss remain elusive.

We established that sall1a, one of the two zebrafish paralogues of the human SALL1 gene transcription factor, is expressed in differentiated hair cells in the inner ear from 48hpf onwards after the expression of brn3c, suggesting that it might be involved in hair cell maturation.

As a means of assessing the involvement of sall1a in hair cell maturation, we performed two different gene-editing and gene-modulating strategies in parallel in a brn3c: GFP zebrafish transgenic line. In our first approach, we co-injected two CRISPR/Cas9 guides flanking the sall1a zinc-finger domains in 1-cell stage embryos in order to generate a knockout. As for our second approach, we designed two CRISPR/Cas13 guides targeting the mRNA and co-injected them to generate a knockdown in sall1a expression. The injected embryos were stained with the vital dye DIASP, which is uptaken by functional and mature hair cells, and performed confocal in vivo imaging at different timepoints. Later on, we compared the numbers of both GFP+ and DIASP+ hair cells between injected and control embryos.

Both approaches showed a significant decrease in GFP+ and DIASP+ hair cells in comparison to controls, which was more pronounced in the case of the CRISPR/Cas13 injection. This might be indicative of an earlier action of this system, which, in contrast to CRISPR/Cas9, can also target maternal mRNA, which is essential during the first hours of embryo development. Moreover, it hints to sall1a holding a role in the maturation and maintenance of hair cells in the auditory and vestibular systems. We are currently performing
transcriptomic analysis of purified hair cells in control and sall1a knockout embryos to uncover the target genes that play a role in hair cell differentiation and function.

Altogether, our study highlights the relevance of using zebrafish for modelling complex diseases in the development of sensory systems, the broad availability of genetic tools for editing and transcriptional modulation and the ease and convenience for performing functional testing in vivo.

Poster Presentations

Title

Identification of compounds involved in habenular circuit development and function

Presenting Author

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Content

Bilateral clusters of habenular neurons in the forebrain of vertebrates relay cognitive information into the interpeduncular nucleus and the median raphe in the ventral mid- and hindbrain, respectively. This neurotransmitter system has been implicated in behaviours from fear and social behaviour to reward responses and addiction. It is also linked to pathophysiological syndromes such as major depression disorder, autism and schizophrenia. Our studies in zebrafish have revealed that the Wnt/beta-catenin signalling pathway gene Tcf7l2 and the precise temporal regulation of the pathway via Wif1 (Wnt inhibitory factor 1) is pivotal for correct habenular neuron differentiation and laterotopic segregation of habenular efferent axons in the IPN target. This knowledge enables us now to generate fish with defined aberrations in the habenulae for analysing the impact on behavior. Intriguingly, Tcf7l2 and Wif1 have been linked to schizophrenia and autism paving the path for further exploring the link between molecule, neural circuit and pathophysiological syndrome. We apply in vivo high-throughput screening (HTS) to identify candidate compounds 1.) impacting habenula development and habenular neuron differentiation and 2.) having an ameliorating effect on habenular neuron malformation and malfunction.

Up to now we screened 160 compounds from 3 different libraries and identified 6 promising candidates showing an effect on habenula development. We will present a detailed analysis of one of the identified compounds. Transient treatment with this particular compound leads to an abolishment of habenular markers, an abundant reduction of habenular neurogenesis and axogenesis and the induction of apoptosis of a specific subset of habenular neurons. Our current work aims at elucidating the underlying mechanism, the signaling pathway affected and the behavioral consequences of this specific phenotype.

Title

The influence of TrkB inhibitor ANA-12 on anxiety in the zebrafish Tuberous Sclerosis Complex model

Presenting Author

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Content

Keywords: zebrafish, Tuberous Sclerosis Complex, TSC, anxiety, TrkB

Tuberous Sclerosis Complex (TSC) is a developmental disease caused by mutations in genes encoding the TSC1 or TSC2 protein, which together form a complex that negatively regulates mechanistic/mammalian target of rapamycin complex 1 (mTORC1). Proper mTORC1 functioning is critical for neuronal development, and main neurological manifestations of TSC include benign brain tumours, childhood epilepsy, and TSC-associated neuropsychiatric disorders, such as intellectual disability or anxiety. Our previous studies have shown abnormal brain morphology, disrupted axonal development, epilepsy, and anxiety-like behaviour in the Tsc2-deficient zebrafish, which correspond to the symptoms displayed by human patients. We have demonstrated a rescue of brain disconnectivity and anxiety-like behaviours by pharmacological inhibition of tyrosine receptor kinase B (TrkB); here, we aim to further explore the link between TrkB signalling, anxiety, and brain development in the pathology of TSC.

In all experiments, wild-type, heterozygous and homozygous Tsc2-deficient zebrafish were treated with the TrkB inhibitor ANA-12, which was previously proven to reduce anxiety-like behaviour. Western blot and immunofluorescence analysis have shown no significant effect of ANA-12 on mTORC1 activity, as measured by the phosphorylation of its downstream target, the ribosomal protein S6, suggesting no direct link between TrkB signalling and the mTORC1 pathway. According to immunofluorescence data, ANA-12 also does not affect the expression or localization of TrkB in the brain, but instead reduces the levels of phosphorylated (active) TrkB, as demonstrated by ELISA assay.

Live imaging of neuronal activity in the habenulae, a brain region implicated in the regulation of fear behaviours and anxiety, was performed in zebrafish at 4 days post-fertilization using a fluorescent GCaMP5G probe. This analysis has shown changes in activity between the Tsc2 genotypes, as well as between fish treated with ANA-12 and an untreated control group. Since the lateral habenula is known to control populations of neurons in the ventral tegmental area (VTA), a region involved in the regulation of mood and sensorimotor control, we have also performed immunofluorescence stainings of the zebrafish VTA homologue, posterior tuberculum (PT). There, we have seen a reduced amount of TH-positive neurons in the Tsc2-deficient fish. Moreover, these neurons exhibited impaired axonal tract fasciculation in the Tsc2-deficient fish compared to wild-type and heterozygous fish, and we are planning to examine the potential effects of ANA-12 treatment on the developmental abnormalities in the PT.

Taken together, our findings point to TrkB signalling as one of potential regulators of anxiety in TSC, affecting brain development, neuronal activity, and behaviour. This underscores the importance of in-depth analysis of the zebrafish TSC model, in order to utilise its full potential as a tool for unravelling the molecular and developmental mechanisms of TSC pathogenesis, and for discovering potential novel drug targets for the treatment of TSC-associated disorders.

Poster Presentations

Title

TRICLOCARBAN AFFECTS MORPHOLOGICAL TRAITS OF EYES AND KEY EXPRESSION GENES FOR EYES DEVELOPMENT IN ZEBRAFISH

Presenting Author

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Content

Keywords: Triclocarban, emerging pollutants, microphthalmia.

Human activity is responsible for producing several chemical compounds, which contaminate the aquatic environment and adversely influence the survival of aquatic species and indirectly human health. Triclocarban belongs to the category of emerging pollutants and its presence in aquatic environment is justified by its wide use as antimicrobial agent in personal care products. The concern about this chemical is due to the risk of persistence in water and soils and its endocrine-disrupting effects. The present study evaluated the developmental toxicity of Triclocarban in zebrafish early-life stages starting with the assessment of acute toxicity and then focusing on the integrative analyses of the observed phenotype on zebrafish development. For this purpose, lethal and sublethal alterations of zebrafish embryos were investigated by the Fish Embryo Acute Toxicity Tests (FET tests). Subsequently, two concentrations of TCC were used to investigate the morphometric eyes features in larvae: an environmentally relevant (5 µg/L) and toxicological (50 µg/L), derived from the No Observed Effect Concentration (NOEC) value concentration. Furthermore, the expression levels of a key transcription factor for eyes differentiation such as mitfb (microphthalmia-associated transcription factor b) and pax6 (paired box protein 6) were evaluated.
The results showed that Triclocarban can alter larvae eyes phenotype and influence the expression of two master genes involved in eyes differentiation, prompting us to further investigate on a possible correlation with its thyroid-disrupting effects and eyes development.

Poster Presentations

Title

Development and Characterization of an Oct1-Deficient Zebrafish Mutant

Presenting Author

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Content

Keywords: organic cation transporters, CRISPR-Cas9, immunohistochemistry, antibody development

Organic cation transporter 1 (Oct1) belongs to the Slc22 family of membrane transporters. It plays an important role in the uptake of a variety of xenobiotic compounds as well as in the maintenance of homeostasis through the transport of endogenous substrates such as steroid hormones, neurotransmitters, and various metabolites. In humans and other mammals, organic cation transporters are expressed on basolateral membranes of toxicologically and metabolically relevant organs such as the liver, kidney, and intestine, and their transport activities contribute to the defense against harmful environmental agents. Similarly, in zebrafish (Danio rerio), oct1 is highly expressed in the kidney and liver of adult animals, and its expression dynamics increases throughout embryonic development, coinciding with the development of these organs. In our previous studies, we identified Oct1-mediated transport of several fluorescent substrates, which provided the basis for the development of transport inhibition assays that demonstrated potent interactions of Oct1 with endogenous steroid hormones and various pharmaceuticals and environmental pollutants. In this study, we present the development of an Oct1-deficient zebrafish line. Using CRISPR-Cas9 technology, we developed an sgRNA guide targeting the first exon of Oct1. This resulted in a deletion of 50 bp in the target exon and a frameshift in translation with a premature stop codon. During development of the homozygous mutant zebrafish, we did not observe a lethal phenotype. To detect the Oct1 transporter at the protein level, we also developed two peptide-based polyclonal antibodies with corresponding epitopes at the N-terminal extracellular loop and the C-terminal cytoplasmic end of the Oct1 protein. Initial immunohistochemical characterization of the developed antibodies confirmed their specificity in the stable FlpIn 293/drOct1 cell line and in the pronephros of 3 days post fertilization embryos. In cryosections of adult zebrafish organs, we confirmed localization of Oct1 in liver and kidney, which was consistent with the previously determined expression at the transcript level. The specificity of Oct1 antibodies was also confirmed in homozygous Oct1-deficient embryos that showed a lack of signal in developing pronephros. The developed Oct1-deficient mutants and the specific Oct1 antibodies provide the basis for future detailed in vivo elucidation of Oct1 function in zebrafish. Further research will focus on detailed characterization of the phenotype of Oct1-deficient mutants by performing exposure experiments with different Oct1 substrates and monitoring the developmental and/or toxic effects on embryos as well as the effects on molecular defense mechanisms in adult zebrafish mutants.

Title

Characterization of endogenous zebrafish melanoma interstitial EVs and their ncRNA content

Presenting Author

Federica Busi

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Content

Extracellular vesicles (EVs) are membranous particles released by all cell types. Their role as functional carriers of bioactive molecules (e.g. RNA, DNA, lipids and proteins) is boosted in cancer where they can be either secreted in biological fluids (circulating EVs) or found in the intercellular space (interstitial EVs, iEVs). Here we have optimised a method for the isolation and characterization of zebrafish iEVs from whole melanoma tissues. Zebrafish melanoma iEVs, characterized by nanoparticle tracking analysis (NTA), have a size range of ~ 140 nm. TEM analysis showed that these particles resemble EVs both in size and structure. Western blot analysis for CD63 and Alix revealed enrichment of these exosomes-related markers in the iEV fraction, but not in melanoma cell lysates. Super resolution microscopy (in TIRF mode) and confocal microscopy revealed that purified zebrafish iEVs were GFP+ indicating that they likely integrate the oncogene, GFP-HRASV12G, used to generate the melanoma model, within their vesicular membrane. Then, we characterized the RNA content of iEVs in comparison with the melanoma of origin. The analysis of the RNA-Seq data revealed that 118 ncRNAs are differentially distributed between zebrafish melanoma and their iEVs, with only 18 of them being selectively accumulated in iEVs. Among these, two RNA components of RNAse MRP and P, which process ribosomal RNA precursors, mitochondrial RNAs and some mRNAs, were enriched in EVs. We then tested if melanoma iEVs induce an inflammatory response by injecting them in zebrafish and found an increase of macrophages and an induction of Interferon Responsive Genes, IRGs. To clarify whether MRP and P contribute to the inflammation induced by melanoma iEVs we injected larvae with MRP or P RNAs and found an inflammatory response similar to that induced by melanoma iEVs. This suggest that zebrafish melanoma iEVs are a source of MRP and P RNAs that can trigger inflammation in cells of the tumor-microenvironment.

Title

Modelling in zebrafish of carnosine/transthyretin interactions in physiology and pathophysiology of familial amyloid polyneuropathy

Presenting Author

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Content

Keywords: transthyretin, carnosine, familial amyloid polyneuropathy, rare disease, zebrafish

Introduction and aims

Familial Amyloid Polyneuropathy (TTR-FAP) is a rare disease caused by mutations of transthyretin (TTR) inducing amyloid formations in peripheral nervous system and cardiac tissue, provoking polyneuropathy, cardiomyopathy, and autonomic dysfunction. Among molecules interfering with amyloid formations, the dipeptide carnosine (CAR, beta-alanyl-L-histidine) is studied as inhibitor of amyloid formations e.g. the Abeta42 peptide in Alzheimer's disease.
CAR is physiologically present in vertebrates' excitable tissues, depending on both dietary intake and endogenous synthesis/degradation/transport processes. Furthermore, CAR homeostasis is intertwined with homeostasis of essential ions including copper, which is in turn investigated with respect to aberrant aggregation of Abeta peptides, prion proteins and, also, TTR amyloid formations.
On these bases, this work is part of a research on physiological and pathophysiological interactions between CAR and wild type/aberrant forms of the TTR protein. Focusing on the Phe84Leu mutation causing TTR-FAP amyloidosis in a specific Italian cohort of patients, the research contemplates the comparative approach based on disease modelling in zebrafish (Danio rerio), for shedding light on conservation of CAR/TTR interaction network in health and disease.

Methods

Comparative and phylogenetic analysis of TTR protein sequences has been performed by aligning the human amino acid sequence (NCBI Acc.No. NP_000362.1) with other vertebrate sequences with NCBI BLAST. Sequence alignment was analysed by Clustal Omega (ClustalO, EMBL-EBI). Protein-protein interaction network and functional enrichment were analysed by STRING. Transcriptional expression of zebrafish ttr gene and of CAR-related genes was investigated by quantitative real time RT-PCR on RNA extracted from selected tissues from adult fish, and from embryos/larvae during development up to 7 days post fertilization, grown in the absence or presence of CAR in the maintenance water. CAR/TTR Molecular docking analysis has been carried out through processing the 3D model of the human protein structure (from PDB archives); the structure was processed by Chimera software. For the zebrafish ttr, structure was predicted by the online tool AlphaFold2. Docking was performed with MOE (Molecular Operating Environment) software, evaluating the binding energy (S, kcal/mol) and the root mean square distances (rmsd, Å).

Findings and conclusions

Here, we report preliminary data for understanding CAR/TTR interactions and for generating the disease model in zebrafish. We performed a comparative/evolutionary analysis of TTR protein sequence, underlining the amino acid residue subject to Phe84Leu mutation. By real time PCR, in adult zebrafish we identified tissue districts of interest regarding expression of ttr gene products, and in embryos/larvae we found, remarkably, that ttr mRNA expression is responsive to CAR treatment during development. By STRING analysis we designed a CAR/TTR minimal interaction network between CAR homeostasis genes and genes functionally related to TTR, in human and zebrafish. Lastly, through molecular docking analysis we compared intermolecular/energy dynamics between CAR and wild type/mutated TTR, both for the human and the teleost protein. These initial data allow optimization of a prototypical approach for diagnostics, nutraceutical and pharmacological investigations for studying the TTR-FAP/Phe64Leu phenotype in zebrafish to provide innovative preclinic support targeted to a specific cohort of the rare disease.

Poster Presentations

Title

Effects of sialylated milk oligosaccharides microinjection on zebrafish larvae survival, locomotion behavior and gene expression

Presenting Author

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Content

Milk is the primary source of nutrients and nutraceuticals compounds for mammal newborns and it represents the gold standard for their initial feeding. Milk oligosaccharides are a class of carbohydrates acting as bioactive factors in numerous defensive and physiological functions. Moreover, they contribute to the development of nervous system and cognitive abilities. Milk contains two classes of oligosaccharides: neutral, if fucosylated at one end, and sialylated, if a sialic acid is present as terminal residual. Since early nutrition can modulate nervous system development and can lead to epigenetic imprinting through changes on gene expression patterns, the intent of the study was to increase the sialylated oligosaccharides content of the zebrafish yolk, with the aim to evaluate the short-term effects on larvae mortality, locomotion behavior and gene expression. Human and bovine milk samples were used to obtain the milk oligosaccharides fractions necessary for the study, a mixture of 3'-sialyllactose and 6'-sialyllactose. Treated embryos were microinjected into the yolk at 4.7 hours post fertilization (hpf) with 4.6 nL of Danieau saline solution or sialylated oligosaccharides solutions, at doses equal to 100 mmol/L. Larvae survival rates were high for all the zebrafish groups (>80%) and no significant differences were highlighted among untreated and treated larvae. The following behavioural endpoints were measured: coiling activity at 30 hpf, locomotion (distance and velocity) and thigmotaxis behaviour at 120 hpf. Locomotor activity and thigmotaxis behaviour were measured even under different contexts (lights ON vs. lights OFF). Coiling analysis showed that burst activities were similar between groups. Locomotion data showed that during the light phase, both distance moved and velocity were found to be similar among control and treated fish. However, when lights were turned OFF, larvae treated with either human or bovine oligosaccharides showed an increased test plate exploration, with longer distance moved and faster velocity compared to control group. Thigmotaxis results showed no significant differences among controls and treated groups, in both light and dark conditions. Total RNA was extracted from untreated and treated larvae and RT-qPCR and RNA-Seq were performed. Real time analyses in treated-larvae suggested a significant decrease of expression levels of zf-gne and zf-st8siaIV mRNAs, two genes involved in sialic acid metabolism. RNA-Seq analyses showed that both treatments affected lipid metabolism. Moreover, bovine milk derived oligosaccharides, seemed to affect neuronal maturation, with an upregulation of synaptic proteins as calcium channels and glutamate receptors, whereas human milk derived oligosaccharides appeared to influence the regulation of circadian rhythms. In conclusion, our study supports the beneficial effects of sialylated milk oligosaccharides administration on zebrafish and it confirms the usefulness of this teleost model as a toll for neuroscience research.

Title

Identification of a new candidate gene in the insurgence of leukodystrophies

Presenting Author

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Content

Leukodystrophies are a heterogeneous group of genetic disorders characterized by degeneration of the white matter in the brain, resulting in motor disabilities, muscle rigidity and even mental retardation and altered sight and/or hearing. Even though around 50% of cases were associated with specific gene mutations, the etiology of the remaining 50% is still unknown. In this work we took advantage of the zebrafish model to assess the pathogenicity of the mutation of a new candidate gene (hereafter gene A), identified in patients affected by leukodystrophy. By both injection of gene A-specific morpholino (gene A-MO) and CRISPR/Cas9 targeted mutagenesis of the zebrafish gene A orthologue, we observed that gene A loss-of-function in zebrafish embryos resulted in a leukodystrophy-like phenotype, characterized by cephalic defects and reduced or absent touch-evoked response. Moreover, both RT-qPCR and whole-mount in situ hybridization analysis indicated a reduction in the expression of genes involved with myelination, namely oligodendrocyte transcription factor 2 (olig2) and myelin basic protein (mbp), and ultrastructure analysis by transmission electron microscopy revealed alterations in myelin formation. Interestingly, morphological, molecular and ultrastructural defects in gene A-MO embryos were rescued by co-injection with zebrafish wild-type, human wild-type but not human mutated gene A full-length transcripts. Our results strongly suggest a functional role for gene A in the myelination process that could be impaired in leukodystrophies. Thereby, future studies will be aimed at assessing more in depth its role in order to better understand the pathomechanisms underlying leukodystrophies insurgence.

Title

Generation and characterization of ap1s2 mutant lines in Danio rerio

Presenting Author

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Content

Keywords: intracellular vesicular traffic, CRISPR/Cas9 technology, neurodevelopment.

In eukaryotic cells, adaptor proteins are complexes that assemble on the cytosolic face of membranes to promote cargo sorting. In particular, the adaptor protein complex 1 (AP-1) is associated with the trans-Golgi network and early or/and recycling endosomes and is composed of four subunits (gamma1, beta1, mu1 and sigma1). Although deficiency for the ubiquitously expressed subunits gamma1 and mu1A is embryonic lethal, knockout mice of sigma-1B isoform (encoded by the Ap1s2 gene) are viable, but reveal impaired tissue functions. Mutations in the human orthologue AP1S2 cause the Pettigrew syndrome (MIM:304340), characterized by mental retardation and additional variable features, including choreoathetosis, hydrocephalus, Dandy-Walker malformation, seizures, and iron or calcium deposition in the brain. Our work has as main objective that of integrating previous knowledge obtained in cellular and animal models through the generation of zebrafish knockout lines for the ap1s2 gene, which encodes the sigma-1B subunit of AP-1.
Microinjection of guide RNA and Cas9 protein was performed in wild-type AB embryos at one-cell stage. Founders were selected using a PCR-based genotyping approach and crossed with wild-type AB fish. Once reached the sexual maturity, F1 heterozygotes were crossed to obtain the F2. Heteroduplex mobility assay and Sanger sequencing allowed to isolate four mutant lines: delta5, delta6, delta7 and delta27. Deletions of 5 and 7 nucleotides in ap1s2 coding region are predicted to cause a frameshift, leading to either the production of non-functional proteins or the activation of the nonsense-mediated mRNA decay mechanism, while deletions of 6 and 27 nucleotides may represent hypomorphic mutations where the altered gene product could maintain a residual biological activity. We decided to initially characterize the ap1s2delta5 line, for which -/- embryos maintain the expected Mendelian ratio at 5 dpf.
Spatial-temporal expression was analysed using an antisense RNA probe for ap1s2: preliminary experiments on wild-type AB embryos showed a strong expression in the central nervous system at 24 and 48 hpf, at the level of the midbrain-hindbrain-boundary, which resulted to be weaker in 48 hpf -/- embryos, probably due to nonsense-mediated mRNA decay mechanism. The effect of ap1s2 deficiency during neurogenesis was investigated by in situ hybridization technique using antisense RNA probes for relevant neural markers (e.g. ngn1 and neurod1), showing alterations in -/- embryos compared to wild-type, e.g. in the cranial ganglia and in midbrain-hindbrain-boundary region.
In silico analysis revealed the presence of a second putative orthologue of the human AP1S2 gene in zebrafish, zgc:162858, which is expressed at a much lower level than ap1s2 during development (RNA-Seq data). We have excluded that ap1s2 mutant F1 lines also carry mutations in this paralogue gene. Thus, ap1s2-/- individuals might express (although at a low level) a functional sigma-1B subunit in some tissues and/or at some developmental stages. Additional studies are required to analyse the biological role of zgc:162858.
Further investigations should include western blot and immunofluorescence, histological sections of the zebrafish brain showing the organization of tissues in the ap1s2 mutants and behavioural studies to verify the presence of alterations of the locomotor system or the memory tasks.

Title

Evaluation of the effects of diets enriched with carnosine and glyciylproline dipeptides administered to adult zebrafish

Presenting Author

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Authors

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Content

Keywords: dipeptides, carnosine, glycylproline, nutrition, zebrafish

In fish nutrition, proteins are the most representative element in dietary formulations meeting nutrient requirements. The intestinal absorption of small-sized peptides, both as protein degradation products and as naturally occurring molecules, is frequently observed to be of greater quantitative significance than the uptake of intact proteins or, even, than free amino acids. Because of their involvement in crucial endogenous physiological processes in specific anatomical districts, e.g. skeletal muscle and connective tissue, the two dipeptides carnosine (beta-alanyl-L-histidine, CAR) and glycylproline (GlyPro) have been identified as dietary substrates potentially exerting ameliorative "nutraceutical" properties, with improvement of nutrient metabolism. In this work, we aimed at evaluating basic effects and physiological variations of diets enriched with CAR and GlyPro dipeptides administered to adult zebrafish, with a focus on gene expression activation at intestine level.
For this, adult zebrafish (> 1 years aged) were administered for 14 days with three different concentrations (0.5%, 1% and 2% w/w) of CAR- and GlyPro- enriched diets. Before (t0), at 7 (t1) and 14 (t2) days post continuous administration, morphometric parameters were evaluated (length, weight and condition factor). Then, treated and control zebrafish were anesthetized and intestines, among other tissues, were sampled and subsequently used for multiple mRNA expression and histological analyses.
The morphometric analysis of length and weight showed no differences between treated and control groups, whereas the condition factor analysis at t2 showed significant higher values for CAR-enriched diets in comparison to the control by all concentrations, indicating allocation of growth based on weight gain rather than on length (i.e., fat fish vs. lean fish). Interestingly, the analysis of length and weight trends for the GlyPro diet along the 14 days of administration showed remarkable differences with respect to control at t1, which disappeared 7 days later, suggesting possible "boosting" effects at short-time. Focusing on the dietary effects at intestinal level, morphometric calculation of villi length showed, interestingly, stimulatory impact (i.e. improvement of the absorptive intestinal surface) both for CAR and for GlyPro diets. Also, at intestine level the mRNA fold change (relative to the untreated control) of a selected network of genes involved in interplaying pathways such as inflammatory states, di- and tripeptides absorption, glucose metabolism, intestinal luminal sensing and gut-brain axis was evaluated. Among all genes, the analysis promisingly showed absence of pro-inflammatory onsets (by analysis of gata4 and nfkb1 marker mRNAs) and, remarkably, enhanced mRNA expression of the glucokinase regulator (gckr) in the CAR- and GlyPro-treated groups, suggesting inhibition of glucose metabolism, probably affecting the mechanism of food intake.
In conclusion, these preliminary results hint significant suitability of CAR and GlyPro for being adopted as nutraceutical dietary components in fish nutrition, that could be exploited for research and development of functional diets meeting nutrient requirements for the zebrafish animal model. This might contribute to the standardization of diets for the zebrafish model in nutritional studies and in physiology research.

Title

Dinitroaniline herbicide pendimethalin affects development and induces biochemical and histological alterations in zebrafish

Presenting Author

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Content

Keywords: zebrafish, pendimethalin, fish embryo toxicity test, Lethal Concentration 50, sublethal effects

Pendimethalin is a dinitroaniline preemergent herbicide widely used to control grasses and weeds. Pendimethalin has been found in agricultural areas where it was not applied, demonstrating the ability to be subject to medium-range transport through the air with consequent deposition in open waters. The detrimental effects of pendimethalin on non-target organisms have been reported above all for the aquatic biota. However, the toxic effects of this herbicide on the zebrafish model have been poorly investigated. The present study aimed to evaluate the pendimethalin potential effects on the development of zebrafish early-life stages. The research focuses first on the acute toxicity, testing 0.25, 0.5, 1, 2, and 3 mg/L of pendimethalin, followed by toxicological results integration through histopathology, oxidative status, and neurotoxicity evaluation using sublethal and environmentally relevant concentrations (0.5 mg/L and 0.05 mg/L). Zebrafish larvae exposed to pendimethalin showed mortality and developed sublethal alterations including impaired fin development, lordosis, scoliosis, blood congestion, impaired blood flow, and reduced heartbeat. At 0.5 mg/L pendimethalin exposure affected musculoskeletal development leading to delayed and reduced ossification of the vertebral centra in the developing vertebral column and disruption of muscle morphology. Herbicide exposure at environmentally relevant concentrations led also to biochemical changes of antioxidant enzymes, increasing the catalase, glutathione reductase and glutatione perossidasi activity, while no effects were observed in the superoxide dismutase and glutathione s-transferase activity. Lastly, acetylcholinesterase activity, was also increased in zebrafish larvae exposed to 0.5 mg/L of pendimethalin. These results confirm the developmental toxicity of in zebrafish early-life stages, pointing out the potential role of oxidative stress in the onset of zebrafish in toxicology research.

Poster Presentations

Title

The effects of 1,3-1,6 ß-glucan in aging using Nothobranchius furzeri as animal model

Presenting Author

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Content

The short-lived annual fish Nothobranchius furzeri has an extremely short life span and accelerated expression of age markers. This model animal makes it particularly suited for investigating the effects of treatments on longevity and age-related pathologies.
The treatment used in this study comes from the cell wall of Saccharomyces cerevicae and is called 1,3-1,6 ß-glucan. 1,3-1,6 ß-glucans modulate immune system by modifying phagocytic and autophagic activity. Also, 1,3-1,6 ß-glucans have antioxidant, anti-inflammatory, and antineoplastic activity. For these reasons, we test the effects of 1,3-1,6 ß-glucans on age-related markers in Nothobranchius furzeri.
From 2 weeks post hatching until 27 weeks post hatching, Nothobranchius have been fed with 1,3-1,6 ß -glucans. 1,3-1,6 ß -glucans were included in a commercial feed in two different doses, a lower concentration (12,5 mg/Kg) and a higher concentration (125 mg/Kg).
Significant results showed that 1,3-1,6 ß-glucans decrease lipofuscin and muscle fibrosis. Moreover, 1,3-1,6 ß-glucans increase autophagy in different organs, as liver and brain. At the same time, the higher concentration of 1,3-1,6 ß-glucans leads to renal toxicity. Indeed, we observed a greater dilation of the renal tubules and an increase in the incidence of precipitation.
In conclusion, 1,3-1,6 ß-glucans seem to slow some age-related markers. This suggests a possible dietary use of ß-glucans to reduce age-related risk.

Title

Temperature effect on BDNF expression in adult zebrafish (Danio rerio): brain proteome and behavioural analysis.

Presenting Author

Mattia Toni

Authors

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Content

Keywords: BDNF, temperature, zebrafish, proteomic, behaviour

Recently, studies conducted by our research group in adult zebrafish (Danio rerio) showed that both acute (4 days) and chronic (21 days) exposure to low (18° C) and high (34° C) temperature alters behaviour and cerebral proteomic compared to 26° C (Toni et al., 2019; Angiulli et al., 2020; Nonnis et al., 2021). Since environmental stress conditions alter the expression of brain derived neurotrophic factor (BDNF) in mammalian models, we investigated the effect of chronic exposure at 34° C on BDNF expression by qRT-PCR and observed a reduction compared to controls, suggesting that it may be involved in the behavioural and proteomic variations previously observed. To further investigate the role of BDNF in the nervous system of zebrafish, we kept wild type, heterozygous BDNF+/- and knock-out BDNF-/- zebrafish at 26° C or 34° C for 21 days. Then, some individuals were euthanized for the analysis of the brain proteome by a shotgun label free proteomic approach and others were subjected to behavioural evaluation by Y-maze test, novel tank test, light and dark test, social preference test and mirror biting test that are behavioural paradigms used for assessing boldness, anxiety, social preference, aggressivity and exploration. Results showed both genotype- and temperature-dependent effects on brain proteome and behaviour. The genotype effect mainly consisted in altered or reduced expression of brain proteins involved in transcription, translation, protein folding and degradation in heterozygous and knock-out zebrafish. Moreover, a down-regulation of proteins associated with energy metabolism, synaptic vesicles, neurotransmitter-mediated signal transduction and cell cycle was observed in knock-out. The behavioural analysis showed an anxiolytic effect of both high temperature exposure and reduced BDNF expression revealing the reduction of freezing events, meandering values, time spent at the bottom of the tank, time spent in the dark compartment. However, the conspicuous alteration of the cerebral proteome could result in an altered perception or processing of external stimuli resulting in a bolder tendency that could be misinterpreted as reduced anxiety. Thus, the increase in straight and non-exploratory swimming in the Y-maze test, the greater exploration of top and light areas respectively in novel tank test and light dark test together with the reduction of bites in the mirror biting test and the reduced modulation of environmental exploration in Y-maze test, may correspond to inability to process the characteristics of the environment and recognize them as dangerous and not to an enhanced boldness and reduced anxiety. This is the first study on an adult BDNF knock-out vertebrate.

Angiulli E, Pagliara V, Cioni C, Frabetti F, Pizzetti F, Alleva E, Toni M. Increase in environmental temperature affects exploratory behaviour, anxiety and social preference in Danio rerio. Sci Rep. 2020, 25;10(1):5385.

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Poster Presentations

Title

Effects of Benzo[a]pyrene (Bap) on the GnRH neuron function in the in vivo zebrafish model

Presenting Author

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Content

Among the polycyclic aromatic hydrocarbons (PAHs), Benzo[a]pyrene (Bap) is considered a common endocrine disrupting chemical (EDC) with mutagenic and carcinogenic effects.The so-called “endocrine disruption hypothesis” suggests that exposure to EDC during fetal, neonatal and adult life may interfere with the development of hypothalamo-pituitary-gonadal (HPG) axis and function of reproductive organs, altering semen quality and reproductive hormone production. Recently, studies performed in different animal models, including zebrafish, described the adverse EDC effects on reproductive outcomes, but the effects of Bap on human GnRH (gonadotrophin-releasing hormone) system are far to be clarified. In this work, we take advantage of the vertebrate zebrafish model to investigate the effect of the exposure of Bap on HPG-axis function, using the reporter line tg(GnRH3:EGFP) that express the green fluorescence under the control of the GnRH3 promoter. The GnRH3 neurons start to differentiate and proliferate at the level of the olfactory placode (OP) at 24-30 hours post fertilization (hpf), projecting their axons dorsoventrally towards the pallium and converging at the midline to form the anterior commissure (AC). Around 40-48 hpf, new fibers elongate along the pre-optic area (POA) to innervate the retina (Re) and extend dorsocaudally their axons to reach the hypothalamus (Hy) at 72 hpf. To evaluate the effects of short-term exposure to Bap, we treated zebrafish embryos with increasing doses of Bap from 2.5 to 72 hpf comparing with the control vehicle (1.25% DMSO). Of note, no significant changes in the survival rates nor in morphological defects have been observed in embryos treated with 5 and 50nM of Bap. Regarding the analysis of GnRH3 network, we observed a dose-dependent reduction or absence of GFP signal at the level of OP, AC, Hy, POA, and Re. In particular, the fluorescence was normal in OP, reduced in AC and Re and absent in Hy and POA in embryos treated with 5nM Bap, whereas it is undetectable in AC, Hy, POA and Re after administration of 50nM Bap. At this point we excluded that the reduced/absent expression of GnRH3 resulted from and increased apoptosis by immunofluorescence of caspase3 apoptotic marker. At this point we wondered if the reduction/absence of GnRH3 signal could results from defects of differentiation of precursors or proliferation of mature GnRH3 neurons in the OP. So, we are now investigating the effects of BaP on the expression of early genes involved in these processes, and the results will be present during the conference.

Title

Development of in vivo model of HPDL deficiency, a novel protein involved in hereditary spastic paraplegia

Presenting Author

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Content

Keywords: Nervous system disorder, HSP, CoQ10, CRISPR/CAS9, Zebrafish KO

Mutations in HPDL, the gene encoding the 4-hydroxyphenylpyruvate dioxygenase-like protein, have been associated with a combination of neurodevelopmental disorders with progressive spasticity, epilepsy and brain white matter abnormalities. However, the function of HPDL and the mechanisms leading to the human disease remain unknown. Bioinformatic and in vitro studies revealed that HPDL localizes to mitochondria, but skin fibroblasts and skeletal muscles from patients did not show significant alterations of oxidative metabolism and mitochondrial dynamic. Hpdl Knock-Out (KO) mice show early lethality (no survival at the stage of P15), display epilepsy, microcephaly, and high cellular apoptosis in the brain, phenocopying symptoms found in a set of patients carrying HPDL variants. However, the early lethality in mice cannot suffice to explore HPDL-related conditions. A more recent polar oxy-metabolome study proposed that HPDL mutations may impair mitochondrial CoQ10 synthesis since HPDL appears to play an unique role in the conversion from the known tyrosine catabolite 4-hydroxyphenylpyruvate (4-HPPA) to 4-hydroxymandelate (4-HMA). Given that reduction of CoQ10 synthesis could result in oxidative stress and mitochondrial dysfunction, the hypothesized combined role of HPDL in neural development and CoQ10 biosynthetic pathway is sound and requires deeper investigations to envisage potential new routes to therapy.
The uncertainty on the protein function and the highest level of expression in the nervous system together with early data obtained in a murine Hpdl-/- model imply that HPDL is an enzyme critical during early brain development and prompted us to study HPDL in zebrafish. The teleost zebrafish has recently emerged as an attractive platform in preclinical research of neurodevelopmental and neurodegenerative disorders placing itself as an important model to bridge the gap between in vitro assays and in vivo studies in mammals. Currently, through CRISPR/Cas9 technology, we have already generated the F0 line. The hpdl-F0 larvae showed motor impairment, reactive oxygen species accumulation and apoptosis, suggesting the potential of the model to replicate the findings in the KO mouse model. The aim of our work is to get better knowledge about the functional role of HPDL during brain development and to get a clearer view of the metabolic picture in HPDL disease, with the final aim to design metabolic target therapies.

Poster Presentations

Title

Evolution of neurotrophin-signaling system in Scyliorhinus canicula

Presenting Author

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Content

Neurotrophins are structurally related neurotrophic factors essential for differentiation, survival, neurite outgrowth, and neurons plasticity. Abnormalities associated with neurotrophin-signaling system (from now on referred as NT-signaling) may lead to neuropathies, neurodegenerative disorders and age-associated cognitive decline such as Alzheimer and Parkinson disease. Whole genome sequencing efforts showed that the NT-signaling, including neurotrophin-like ligands, p75NTR, and Trk-like receptors, evolved before the evolution of vertebrates, thus, the shared ancestor of protostomes, cyclostomes and deuterostomes must have possessed neurotrophins, p75NTR, and Trk receptors orthologs. After the first round of whole genomic duplication occurred in the ancient in deuterostomes, the presence of two neurotrophins in cyclostomes was hypothesized. We then performed a phylogenetic analysis including all the neurotrophin sequences deposited in NCBI and ENSEMBL repository for the gnathostomes. On note, the results allow us to identify for the first time the second duplicated neurotrophin in the taxon. Since a second whole genome extra duplication round occurred in cyclostomes during the evolution, we then proceeded to expand the analysis including the group of cartilaginous fishes, or chondrichthyans. The chondrichthyans group is a monophyletic group that represents the out-group to all other living jawed vertebrates (gnathostomes) and the sister group of osteichthyans (comprehensive of actinopterygians and sarcopterygians). The strategic phylogenetic position as one of the most basal existent vertebrates with jaws indicates chondrichthyans as a key group for understanding the evolution of NT-signaling in the brain of vertebrates. Results from the phyletic analysis indeed showed the presence of four neurotrophins in the chondrichthyans, namely the four mammalian neurotrophins Bdnf, Ntf3, Ntf4 and Ngf. The same analysis applied to Trk receptors surprisingly showed in chondrichthyan the presence of two trk receptors only, instead of the three mammalian Trks, raising the hypothesis of a more broad function of p75NTR in mediating the NT-signaling in this specific phyletic taxon. We then adopted the Scyliorhinus canicula as model system to validate our findings. Scyliorhinus canicula samples were collected and intronless neurotrophins bdnf, ntf3, ntf4 and ngf were PCR amplified from genomic DNA. For the Trks and p75 receptors we proceeded with standard RNA extraction, cDNA retrotranscription and PCR amplification. Templates were sequenced to confirm the correct gene amplification and subsequently used to generate probes for in situ hybridization (DIG labeling ISH probes for colorimetric revelation). ISH were then performed on fresh frozen brain sections. Our first results confirmed the presence in the brain of Scyliorhinus canicula of all four neurotrophins, the two Trk receptors and P75, in line with our expectations. Such data showed the importance of Scyliorhinus canicula as a key model system for the study and the understanding of vertebrates NT-signaling during the evolution, potentially unveiling new strategies for the treatment of neurotrophin related diseases. The introduction of Scyliorhinus canicula model system will add new advances that can be exploited in other conventional and unconventional model systems, contributing significantly to the neurobiology of the fish field.

Poster Presentations

Title

Simultaneous stress and D-glucose treatment causes hyperglycemia and induces oxidative stress in adult zebrafish brain.

Presenting Author

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Content

The zebrafish has recently gained popularity as a disease model in addition to its utility in genetic, developmental, and drug screening studies. Researchers have successfully developed the chronic hyperglycemia model in adult zebrafish in an effort to study the pathophysiology of metabolic disorders such as diabetes. It is speculated that chronic diabetic conditions involve considerable physiological and psychological stress, which is termed diabetic distress. It has been revealed that patients suffering from diabetes often experience chronic diabetic distress. Although chronic stress is modeled in zebrafish in the context of psychiatric illnesses such as depression and anxiety, extensive literature addressing the effects of concurrently occurring chronic stress and hyperglycemia is currently lacking. Aiming to bridge this gap, we performed a study wherein adult zebrafish were simultaneously exposed to D-glucose water and chronic unpredictable mild stress (CUMS) for 14 and 21 days. The fishes were divided into 4 groups: control, CUMS, D-Glucose, CUMS + D-Glucose. To induce hyperglycemia, fishes were incubated in D-Glucose solution (111mM) after which fasting blood glucose levels were measured with a glucometer. The CUMS procedure involved exposing the fishes to six stressors: i) restraint (60 mins) ii) overcrowding (60 mins) iii) chasing with a net (8 mins) iv) tank change (6 times in succession) v) elevation over water (2 mins) vi) isolation (60 mins). All of these stressors were applied in a random, unpredictable manner, and each fish was exposed to two different stressors per day. There is mounting evidence that oxidative stress is involved in the pathophysiology of various diseases. We hypothesized that subjecting fishes to chronic mild stress in addition to hyperglycemic conditions would impair redox homeostasis in the brain. In this study, whole brain samples were used for biochemical assays: i) catalase and superoxide dismutase (SOD) activities ii) reduced glutathione (GSH) levels iii) lipid peroxidation (LPO) content. Fasting blood glucose levels were higher in both the D-Glucose and CUMS + D-Glucose groups after 14 and 21 days of treatment. In addition, CUMS + D-Glucose fishes exhibited higher specific catalase activity after 14 and 21 days of treatment while SOD activity was elevated after 14 days of double stress, but was comparable to the control group after 21 days of treatment. The levels of reduced GSH were decreased in CUMS + D-Glucose group after 14 and 21 days of treatment. LPO levels were increased in CUMS + D-Glucose and D-Glucose groups after 21 days but not after 14 days of treatment. In conclusion, simultaneous treatment with chronic stress and hyperglycemia in the adult zebrafish may induce redox imbalance in the whole brain. This has implications for the effect of diabetic conditions (high fasting blood sugar levels) and chronic stress on brain function. Using this double treatment model, zebrafish researchers would be able to further investigate the underlying disease mechanisms of comorbid diabetes and chronic stress, which would open doors for the development of therapeutic drugs that could effectively alleviate disease progression.

Keywords: adult zebrafish, chronic hyperglycemia, chronic mild stress; oxidative stress; blood glucose.

Poster Presentations

Title

Characterization of grna zebrafish morphants treated with the autophagic modulator SG2

Presenting Author

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Content

The Neuronal Ceroid Lipofuscinosis type 11 form (CLN11) is a neurodegenerative, lysosomal storage disorder (LSD) that primarily affects the central nervous system. CLN11 disease is characterized by recurrent seizures (epilepsy), vision loss, problems with balance and coordination (cerebellar ataxia), and a decline in intellectual function due to mutations in CLN11/GRN. GRN encodes progranulin (GRN), a secreted, glycosylated protein involved in liver growth, motor neurons (MNs) development, maintenance of myogenic progenitor cells that likely converge to a common pathway where abnormal lysosomal function leads to accumulation of undegraded metabolites, dysregulation of autophagy and impairment of the intracellular degradation pathway essential for cellular survival and organismal health. Autophagy is perhaps one of the most interesting mechanisms regulating cell homeostasis in many physiological and pathological conditions, and it is associated with a growing number of pathological conditions, especially in the central nervous system. Accumulation of lysosomal lipid were observed in CLN11 patients' skin fibroblasts and in mouse Grn-/- neurons. To investigate the autophagic flux quickly and easily we used a morphant grna zebrafish model that reproduce the CLN11 disease with truncated primary MNs, reduced body length and impaired motor function. In this study, we tested a recently patented (SR, University of Pisa) small molecule, termed SG2, with a pleiotropic activity, including autophagic flux promotion, neuroprotection, and metabolic reprogramming. SG2 has been used in models of defective autophagy and in Alzheimer's disease (AD). This molecule showed positives performances either in vitro, in different AD cell lines, or in vivo, in worms and mice models of AD. The safety profile of SG2 in zebrafish allowed us to test if this small molecule can modulate autophagy in GFP-Lc3 transgenic grna morphants. We performed behavioral tests through the evaluation of the burst activity at 30 hour-postfertilization (hpf) and locomotor performances measuring the distance and velocity travelled. Moreover, we assessed basal and induced autophagic flux in whole organism grna morphants. The results showed an increased burst activity in grna embryos and a reduced locomotion, in terms of distance and velocity in 5 day-post-fertilization (dpf) grna larvae. SG2 treatment reduced the tail coils of embryos in the chorion and it slightly ameliorated locomotion of larvae at 5 dpf; whereas the increased lc3 fluorescence in the morphant group was not modified by SG2 administration. Furthermore, the effect of SG2 on autophagy was evaluated through expression analysis of known autophagy biomarkers by RT-qPCR and western blot. With this study, we set out effectors in autophagic pathway of CLN11 disease that can be pharmacological modulated using zebrafish as animal model.

Keywords: Autophagy, Neuronal Ceroid Lipofuscinosis, Granulin, Zebrafish, SG2

Poster Presentations

Title

The geometry in spatial learning by zebrafish (Danio rerio)

Presenting Author

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Content

Geometric reorientation is a special phenomenon within the research field of spatial cognition in animals and consists in using large-scale environmental attributes to locate a salient place after disorientation. To reproduce in controlled settings such a situation, geometrically typified enclosures have been for long employed: that is the case of rectangular arenas, where attributes of length, distance, and angular magnitude get in touch with left-right directions. The susceptibility to large-scale geometry was suggested after observing that animals consistently confused two goal-positions sharing the same relationships (e.g., the two diagonal corners with a short/long surface on the left/right), both in spontaneous and rewarded reorientation tasks. We were interested at inspecting whether zebrafish (Danio rerio) could learn to reorient through spatial geometry, thereby encoding all the informative attributes provided by a rectangular layout of surfaces. We first tested reorientation behavior of fish within an all-white rectangular arena, to then explore the use of distance, corners, and length, one at a time. To set these attributes apart, reorientation behavior of fish was observed within transparent arenas, where we specially probed the impact of each by handling the global shape of the experimental space (rectangular or square). Along the perimeter: four white surfaces equal in length were used to test the distance attribute; four white C-shape surfaces were used to test the corner attribute; four white surfaces of 2:1 ratio in length were used to test the length attribute. Zebrafish adult males were engaged in a daily training targeted to reward them for swimming across a "corridor of choice" placed at levels of the two corners on the correct-geometry diagonal, until a learning criterion ≥70% per two consecutive sessions (one per day: learning and validation). Results showed that zebrafish learned to approach the correct-geometry diagonal instead of the incorrect-geometry one, thus reorienting through the large-scale geometry provided by the all-white rectangular arena. When dissecting the geometric attributes in fragmented layouts, results showed that zebrafish learned to encode all the geometrically informative attributes, in terms of distance, corners, and length in association with left-right directions (e.g., the two corners on the correct-geometry diagonal had a close/far surface on the left/right). Altogether, our findings: (1) emphasize the role of behavioral protocols (spontaneous versus over-trained choice) on zebrafish reoriented navigation; (2) stress the ecological relevance of large-scale geometry as a source of spatial knowledge; (3) allows targeted comparisons due to behavioral and cognitive similarities among zebrafish and mammals.

Keywords: navigation, spatial learning, geometry, reorientation, zebrafish

Poster Presentations

Title

Generation of a Crestin-based dual-reporter zebrafish line for drug screenings

Presenting Author

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Content

Melanoma is one of the most common skin cancers and is the deadliest, due to its ability to form metastases. In our lab, we study melanoma driven by BRAF substitution V600E. This proto-oncogene encodes for a constitutively active kinase that promotes cells growth and division through MEK/ERK signaling pathway.
In the Tg(mitfa:BRAFV600E);p53-/- transgenic line, the most widely used melanoma model in zebrafish, tumors form in adult fish due to the absence of p53 and to the melanocyte lineage-restricted expression of BRAFV600E. Interestingly, in this genetic context, melanoma cells are characterized by a transcriptional reprogramming that leads to the re-activation of many genes in common with neural crest cells (NCCs). These genes, such as Crestin, are physiologically expressed in embryonic multipotent NCCs and are downregulated after their differentiation, but they get aberrantly re-expressed in melanoma cells in the adult fish.
Our aim is to use embryos of the Tg(mitfa:BRAFV600E);p53-/- and take a decrease in Crestin levels as read-out of efficacy of anti-melanoma drugs. In fact, we have preliminarily confirmed that, once injected with crestin:mCherry or crestin:luciferase constructs at 1-cell stage, 30hpf embryos show lower levels of fluorescent signal or luciferase activity, upon 24h of treatment with BRAF inhibitors (BRAFi).
Currently, we are generating transgenic zebrafish lines expressing a reporter construct that allows to monitor the decrease in Crestin levels qualitatively and/or quantitatively. Specifically, crestin:mCherry and crestin:luciferase single-reporter constructs, as well as crestin:luciferase-P2A-mCherry dual-reporter constructs, express mCherry (as qualitative marker) and/or Luciferase (as quantitative marker), under the control of Crestin promoter. These constructs have been already injected in embryos of the Tg(mitfa:BRAFV600E);p53-/- line and filial generations are growing. Our plan is to select and further use the line that allows to assess, in the most accurate and reproducible way, the decrease in Crestin levels that occur upon various pharmacological treatments.

Keywords:
melanoma, neural crest cells, Crestin, drug screening, BRAFV600E

Title

Generation and characterization of a new zebrafish knockout model for SPG4 Hereditary Spastic Paraplegia.

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Content

The Hereditary Spastic Paraplegias are a miscellaneous group of neurologic disorders characterized by pathophysiologic hallmark of length dependent axonopathy of the corticospinal tracts. The eighty five different spastic gait disease loci and more than eighty spastic paraplegia genes identified so far, made this pathology one of the most genetically heterogenous monogenic disorders. The impairment of several cellular functions such as the axonal transport, the endoplasmatic reticulum modeling, the mitochondria's activity or the lipid metabolism, can be associated with the onset and the progression of the pathology. Moreover, there are growing evidences showing that also the alteration of Bone Morphogenetic Protein signaling pathway is an important molecular process associated with the pathogenesis of such disorder. Recently, zebrafish come up to be an attractive model for studying human genetic disorders and despite the existence of key differences in the neuromuscular system, Danio rerio can be a valuable tool for the molecular and genetic dissection of spastic paraplegia pathogenetic mechanisms in vivo. In view of this, we generated by CRISPR Cas9 a stable knockout zebrafish line for the most common form of Hereditary Spastic Paraplegia associated with mutation in Spastin gene also called SPG4. This new knockout mutant is characterized by a 11 nucleotides insertion in exon 2 of the Spastin gene that alters the open reading frame of the transcript and introduces a premature stop codon in the resulting predicted protein. To strengthen the idea that this mutant line can phenocopy the patients, motility analyses were performed individually in multiwell plates by Noldus automated video tracking system in order to identify the presence of motor impairment. In basal condition, wildtype and mutant spastin fish exhibited similar swimming capacity while after the administration of tunicamycin, an endoplasmatic reticulum stress inducer, spastin homozygous knockout showed a reduced mobility demonstrating a major sensibility to this stress condition than controls, as expected. Interestingly, spastin mutants also showed a dysregulation of the Bone Morphogenetic Protein signaling pathway that resulted overexpressed during the embryonic development. To confirm these data, motor neurons integrity was assessed and the presence of putative malformations investigated by conventional and confocal analyses. Altogether, our preliminary results highlight the possibility to obtain a valid tool toward the exploration of the molecular scenario of Hereditary Spastic Paraplegia as well as a suitable zebrafish model for application in large scale pharmacological screenings.

KEY WORDS: CRISPR Cas9, Bone Morphogenetic Protein signaling, Hereditary Spastic Paraplegia, Spastin

Title

Loss of cyp2u1 leads to increased mortality and early locomotor impairment in zebrafish

Presenting Author

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Content

Keywords: SPG56, hereditary spastic paraplegia, knock-out

Background: Biallelic variants in CYP2U1 cause SPG56 (MIM number 615030), an ultrarare, autosomal recessive form of hereditary spastic paraplegia characterized by early-onset spasticity, macular degeneration, and cognitive impairment with hypomyelination at brain imaging. About 50 patients have been described thus far but additional cases remain unreported. CYP2U1 encodes a member of cytochrome 450 family 2 highly expressed in several districts, including brain, and localized in mitochondria and endoplasmic reticulum, with a known role in lipid hydroxylation, arachidonic acid metabolism, and bioenergetics. Recently, the generation of a murine knock-out (KO) model of SPG56, provided precious insights putting forward the mechanism linking CYP2U1 with abnormal folate metabolism in early disease stages, accompanied by enhanced oxidative stress and impaired mitochondrial energy metabolism. However, the mouse model does nor recapitulate precisely the human condition with mice having normal motor function without features of early deficits and presenting cognitive impairment at the age of 18 months as it occurs late in SPG56 patients. Hence, we aim to provide a novel in vivo model able to better resemble early steps of SPG56, and suitable for future pharmacological treatments.

Methods: CRISPR/Cas9 technology was employed to generate a knock-out strain in zebrafish cyp2u1 orthologue (ENSDARG00000026548, 55% identity). sgRNA was designed to show no predicted off-target effects and injected in wild type AB strain. sgRNA-injected F0 embryos were raised to adulthood, crossed with wild type adults to obtain F1 heterozygous individuals, and those were further crossed to breed F2 homozygous fishes. Experiments were carried out using F3 cyp2u1-/- larvae. RT-qPCR was used to determine cyp2u1 ablation. Survival rate was measured within 120 hpf, coiling frequency was analysed at 30 hpf with DanioScope, whereas locomotor activity was acquired at 120 hpf with DanioVision and measured with EthoVision XT.

Results: We successfully generated a zebrafish cyp2u1-/- strain harboring a 2-bp deletion mutation in exon 2 leading to a premature stop codon (c.688_689del, p.His230Ter). RT-qPCR analysis revealed a nearly complete ablation of cyp2u1 mRNA expression, likely driving to a complete depletion of protein level that we could not measure in the absence of appropriate antibodies. Survival analysis showed early mortality with a significant increase of deceased mutant larvae compared to controls. Defects at early stages of life were also assessed by a marked reduction of coiling frequency at 30 hpf, suggesting the presence of locomotor impairment, that was subsequently confirmed by a significant reduction of motor performances at 120 hpf. Taken together, these data suggest that our model resembles the human loss-of-CYP2U1 driven neuromuscular impairment, hallmark of SPG56.
Conclusions: We generated a promising and reliable model of SPG56, caused by CYP2U1 loss of function. We foresee that it will constitute a powerful alternative to murine Cyp2u1 knock-out recently generated since it resembles the early locomotor SPG56 phenotype, conversely to Cyp2u1-/- mice. This feature makes our model a suitable platform for pharmacological screenings with powerful translational implications.

Title

Identification of regulated mRNAs and miRNAs in glomeruli isolated from a Focal segmental glomerulosclerosis zebrafish model

Presenting Author

Anna Iervolino

Authors

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Content

The zebrafish (Danio rerio) is a powerful model to study glomerular morphology and the function of the glomerular filtration barrier. Since zebrafish larvae develop quickly and can be bred to become transparent, in vivo observation of these animals is possible. At 48 hours post fertilization, zebrafish larvae develop a single glomerulus, which is attached to a pair of renal tubules. Like in mammals, the glomerular filtration barrier consists of a fenestrated endothelium, the glomerular basement membrane and interdigitating podocyte foot processes bridged by a slit diaphragm. By using genetically modified zebrafish strains with fluorescently labeled podocytes, it is possible to induce podocyte apoptosis and detachment from the glomerular basement membrane studying alterations of the glomerulus during the development of renal disease like focal segmental glomerulosclerosis. The aim of our study was to collect glomeruli for the identification of mRNAs as well as miRNAs by RNA_Seq that are up- and down-regulated in the glomeruli of this focal segmental glomerulosclerosis like disease model.

Method: The transgenic zebrafish strain Cherry which expresses the prokaryotic enzyme nitroreductase fused to mCherry, a red fluorescent protein, under the control of the podocyte-specific podocin promoter in a transparent zebrafish strain, was utilized. After addition into the tank water, the metronidazole is converted into a cytotoxin by nitroreductase leading to dose-dependent apoptosis exclusively in podocytes. Treated and control larvae were homogenized at 6 days post fertilization. The cell suspension was diluted, and red-fluorescent glomeruli were collected using a micropipette. Total RNA was isolated, and integrity was checked by a Bioanalyzer. Libraries were generated and constructs were amplified by PCR and sequenced on an Illumina Hiseq 2000. Normalization and statistical analysis for differential gene expression were done using DESeq2.

Results: Treated larvae developed periocular, yolk and pericardial edema. Edema progressively increased in a time dependent way and often associated with increased mortality due to podocyte damage and loss, which is the prerequisite for the development of focal segmental glomerulosclerosis.
In order to perform gene analyses of glomeruli, we established a method enriching glomeruli with an excellent quality. Only the RNA of manually collected glomeruli had an excellent quality. Using RNA_Seq, we identified a total of 16941 genes. DESeq2 analysis showed 494 up-regulated and 473 down-regulated genes. Gene ontology enrichment analysis of up-regulated genes revealed a total of 167 that are significantly enriched in GO terms (metabolic processes, immune response and ion transport). Down-regulated genes were enriched in 14 GO terms and most of them are linked to normal glomerular function. DESeq2 analysis identified 200 miRNAs of 777 small RNAs. Some of these miRNA are already described to be regulated in different glomerular diseases like FSGS, lupus nephritis, IgA nephropathy and diabetic nephropathy.
Conclusion: We analyzed isolated glomeruli from transgenic zebrafish larvae that developed a FSGS-like disease. By sequencing, we have found mRNAs and miRNAs that were significantly regulated after the onset of disease. Detailed knowledge of these mRNAs and miRNA-based gene regulation will help to uncover the pathomechanism as well as to develop therapeutics for the treatment of FSGS.

Title

Impact of maternal and zygotic transcripts on mutant setd5-driven social impairment in zebrafish

Presenting Author

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Content

Loss-of-function (LoF) mutations of SETD5 in humans have been associated with intellectual disability (ID) and autistic spectrum disorders (ASD). SETD5 encodes for a histone methyltransferase, highly expressed in the brain, whose aploinsufficiency leads to a reduced methylation of Lysine 36 of Histone 3 (H3K36). The aim of this study is to characterize SETD5 LoF zebrafish models generated by CRISPR-Cas9 gene editing technique from a genetical, morphological and behavioral point of view. In particular, as Setd5 displays both maternal and zygotic transcripts, we generated setd5 maternal-zygotic knockouts, as well as heterozygous fishes carrying either wild type or mutated maternal setd5 transcripts. Adult brain samples from LoF individuals display reduced expression of genes encoding for synaptic proteins and neurotransmitter metabolism, associated with microcephaly and a significant reduction of body length and eye diameter in both larvae and adults. Furthermore, adult fishes display a significant reduction of telencephalon and optic tectum area. Additionally, setd5 LoF leads to repetitive swimming behavior, altered social interaction and reduced interest for social novelty partially ameliorated by the antipsychotic drug Risperidone. Interestingly, we found that the different mutant maternal contribution greatly impacts the future development of the individuals, since the heterozygous fishes carrying mutant maternal transcripts actually present the worst outcome.
Validating this reliable and easy-to-use model for ASD/ID could represent a fundamental tool for the advancement of therapeutic protocols. The zebrafish setd5 LoF model could be used for the screening of compounds able to rescue the morphological and behavioral effect that, in perspective, could help treat individuals affected by ASD/ID caused by SETD5 haploinsufficiency.

Keywords: Setd5, Zebrafish, ASD, ID

Title

A zebrafish transplantable model for lung carcinoids

Presenting Author

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Content

Lung carcinoids are low/intermediate grade neuroendocrine tumors accounting for less than 1% of all lung cancers. Since these tumors can be clinically silent for long time, they are often diagnosed once metastasis have already occurred. It has been estimated that the 25-30% of cases develops distant metastasis. So far, therapies for these advanced forms are unavailable. In this frame, animal models that faithfully recapitulate clinical features and related complexity of lung carcinoids may help the development of new therapeutic strategies.
In this work, a new experimental model have been developed by the implantation of four human lung carcinoid tumor cell lines (NCI-H727, NCI-H720, UMC-11, NCI-H835) and primary cultures in the subperidermal space of 2 days post fertilization Tg(fli1a:EGFP)y1 zebrafish embryos. NCI-H835, UMC-11 and NCI-H727 displayed a higher proangiogenic potential compared to NCI-H720, leading to the formation of endothelial structures which sprouted from the plexus of sub intestinal vessels and the common cardinal vein as early as 1 day post injection. NCI-H720 cells displayed a higher invasiveness compared to the other implanted cell lines in a time frame of 2 days post injection. Carcinoid cells preserved both histological morphology and proliferation rate after the implantation. Due to the possibility of implanting a limited number of tumor cells to observe a positive pro-angiogenic response and an invasive behaviour, our platform resulted particularly suitable for the engraftment of carcinoid cells derived from post-surgical sample, whose size is often limited.
Our carcinoid xenograft model have been used also to study the anti-tumor effects of three tyrosine kinase inhibitors (axitinib, cabozantinib and sulfatinib), previously tested for other neuroendocrine tumors. A dose-dependent inhibition of tumor-induced angiogenesis was reported after the treatment with each selected drug, whereas only the administration of cabozantinib resulted able to reduce the invasive behavior of implanted cells.
In conclusion, xenograft in zebrafish embryos resulted a cheap and fast platform to study tumor-induced angiogenesis and tumor cell dissemination of lung carcinoids, as well as to perform drug screening. The possibility to implant patient-derived carcinoid cells opens a promising scenario for the identification of personalized therapies.

Keywords: lung carcinoids, tumor xenografts, tyrosine kinase inhibitors

Title

An optimized protocol for regular care of Nothobranchius furzeri in standardized laboratory conditions

Presenting Author

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Content

Keywords:
Killifish, housing, husbandry, basic protocols

Abstract:

In the last few years, the African killifish Nothobranchius furzeri has emerged as an important model system for the study of vertebrate biology. N. furzeri is an annual fish that inhabits seasonal freshwater ponds in the southeast of Africa and is characterized by rapid growth and early sexual maturation. The short median lifespan of 3 and 7 months reflects an adaptation to the ephemeral nature of the habitat. In fact, this fish is currently considered the shortest-lived vertebrate that can be bred in captivity. Importantly, despite its short lifespan, N. furzeri recapitulates typical age-dependent phenotypes and pathologies making this fish a suitable model for ageing research. Rapid growth, maturation and ageing make maintenance in captivity of N. furzeri laboratory strains challenging. Our goal was to optimize available husbandry and housing protocols, with the aim to keep this species in the laboratory environment on a large scale as well as ensure reproducibility of experimental work. Starting from the comparison of the published protocols, we set up detailed notes for eggs incubation, hatching, daily care of juvenile and adult fish, feeding and breeding. We standardized diapause period using only humic acid, avoiding peat and/or other organic substances to reduce sources of contamination and variability. To ameliorate hatching rate, we suggest the use of low temperature of both humic acid and the tank used for hatching. We optimized the feeding protocol of both juvenile and adult fish, by feeding fish three times a day to avoid delay in the sexual maturity, establishing a precise weaning protocol and the quantity of frozen bloodworms eaten by the single fish. For breeding we recommend spawning once per week. With this work we address the different challenging issues associated with the laboratory use of N. furzeri. We believe that the improved housing and husbandry conditions will be of great utility to other groups who want to approach with this species and could help to define killifish welfare guidelines.

Title

Preliminary results on Leptin immunolocalization in Danio rerio ovary

Presenting Author

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Content

Severe reproductive deficiencies in female zebrafishes have been seen using a leptin receptor knockout strain (Tsakoumis 2021). This study aims to reveal Leptin localization in the ovary of wild-type zebrafish to give insights on the role of this hormone on the reproductive physiology of this species.
Four females of Danio rerio – strain AB; age 330 days; Uppsala University – were fasted for 24h and euthanized with an overdose of MS222. The individuals were 0,67 ± 0,10 g in body weight, 3,81 ± 0,24 cm in total length and 3,57 ± 0,23 cm in fork length. The specimens were fixed in 4% formaldehyde solution, decalcified in 0.5 M EDTA, tissues embedded in paraffin and whole body longitudinal or transversal 5 um sections mounted on slides. Dewaxed slides were stained with Hematoxylin-Eosin (HE) or pre-treated using heat mediated antigen retrieval (microwaved with sodium citrate buffer, pH 6.3). The latters underwent endogenous peroxidase blocking (H2O2 3% in distilled water) and were successively incubated with goat serum (1:10) to block nonspecific binding. A primary polyclonal antibody anti-Leptin [sc-842 (Santa Cruz Biotec. Inc., Santa Cruz, CA, USA) against zebrafish Leptin-A following Garcia-Suarez et al. (2017) and Montalbano et al. (2020)] was employed at 1:100 PBS dilution and overnighted at room temperature (a section per slide was used as negative control omitting the primary ab). The following day, interspersed with PBS washes, the slides were incubated with the secondary antibodies (goat anti-rabbit, 1:200) and treated with an avidin–biotin complex to use diaminobenzidine as chromogen.
The HE staining highlighted a vitellogenic oocytes predominance in all the ovaries, suggesting an intermediate stage between postspawning and prespawning (van der Ven & Wester, 2003). Immunoreactivity has been observed only in sections incubated with primary antibodies. The immunohistochemistry revealed the presence of Leptin-A in ovarian cells at late maturation stages. Immunoreactivity was observed in granulosa cells (GCs) starting from the vitellogenic stages and increased through more mature stages: the immunostaining of GCs appeared light in vitellogenic follicles (specifically around late primary and early secondary oocytes – stage 6-7), it was mild/moderate in early postovulatory follicles, while was stronger in late postovulatory follicles (atretic follicles or corpora atretica).
Our results suggest that the Leptin-A presence in zebrafish ovary might follow the trend of follicular atresia, since the intensity of immunoreaction seems to constantly grow from GCs of vitellogenic follicles reaching a peak in hypertrophic and degenerated GCs of corpora atretica. In contrast, immunopositivity was not seen in previtellogenic stages. This immunolocalization is coherent with the results from Tsakoumis (2021), that suggest an important role of leptin in the last steps of zebrafish follicular development, particularly oocyte maturation and ovulation. Nevertheless, Gorissen et al. (2009) showed that leptin-a gene is hardly expressed in zebrafish ovary – where its paralogue leptin-b had the highest expression – and the significance of its translated protein (Leptin-A) in GCs should be ruled-out. Further studies need to be performed to shed light on the relevance of leptin(s) and its receptor in the follicular development and atresia of Danio rerio ovary.

Title

Score sheets for Nothobranchius furzeri: a practical tool for welfare assessment

Presenting Author

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Content

Laboratory fish, animal welfare, pain assessment, humane endpoint, 3Rs

Animal based research needs to strictly adhere to the 3R principle (replace, refine, reduce), for the ethical justification of the use of animals in science as well as to ensure the highest quality of reproducible data. With the implementation of the EU Directive 2010/63 within national law it is mandatory to perform a severity classification of procedures on the basis of estimated levels of pain, suffering, distress and lasting harm that is inflicted on the animals. To standardize and formalize severity assessment, score sheets are commonly in place to record the effect of scientific procedures on animals, and therefore to inform decisions on remedial measures or experiment termination. The goal of our work was to determine a well-designed score sheet for the recently well-established animal model, the African turquoise killifish Nothobranchius furzeri, the shortest-lived vertebrate that can be bred in captivity. In the first step we have identified four parameters that will serve to detect deviations from the normal state: behavior, swim, body condition and clinical signs. Then we determined all possible symptoms that can influence these parameters. For behavior we evaluated aggression manifested towards the other sex, social isolation, food disinterest, lethargy, gasping; for swim we estimated partial or intermittent loss of balance, circling, belly slider, total loss of balance; for body score we considered loss of 10% to 15% of weight, loss of 15% to 20% of weight, emaciation, obesity; as clinical signs we analyzed color alteration, loss of scales, alteration of the operculum, intermittent hyperventilation, cutaneous mucus hypersecretion, exophthalmos, ulcers/neoformations, hemorrhage, skeletal deformations, permanent hyperventilation. In the second step we defined a numerical scoring that goes from 0 to 3 in order to weigh each symptom. As last step, from symptoms sum up we established the interventions that are applied in the event of animal suffering, including humane endpoints. Total score can vary from 0, no action, up to values higher than 8, that represent our humane endpoint implying the euthanasia of the fish. This simple scoring tool can be employed for long-term monitoring of individual animal welfare and integrated into routine management practices. Future step will be to enrich this tool with age-related enhanced monitoring to define (a) the expected onset of one or more age-related adverse clinical signs (depending on the genetic background), (b) the age-related clinical signs that can cover possible pathological conditions, (c) the application of humane end points will help to reduce the numbers of animals maintained for longer than is scientifically justified.

Poster Presentations

Title

Evaluating a potential role for setd5 inactivation in the aging process

Presenting Author

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Content

Numerous lines of evidence support the notion that epigenetic changes directly contribute to aging and aging-related diseases. Deciphering the molecular mechanisms underlying the activity of epigenetic regulators in the context of aging represents therefore a crucial step in the comprehension of this important biological process.
SETD5 is a gene highly expressed in the brain encoding for a methyltransferase whose activity on lysine 36 of histone H3 has been recently demonstrated. Setd5 loss-of-function (LoF), which has been associated with intellectual disability and autistic spectrum disorders, leads to a reduction of H3K36me3, an epigenetic change that has been previously correlated with the aging process.
The aim of this study is to address the potential role of setd5 LoF in premature aging by evaluating specific parameters in zebrafish setd5 mutants. We found that mutant zebrafish individuals display an altered expression in selected genes involved in brain aging. Furthermore, setd5 LoF causes several age-related behavioral impairments, which we assessed through specific behavioral tests. In fact, as setd5 mutant fishes grow older, we observed a decline in social interaction and a reduction of interest for social novelty, which are significantly different from the ones we recorded for wild type fishes.
Additionally, when compared to wild type fishes, mutant young fishes are less responsive to different types of stimuli and show signs of premature aging when subjected to the T-maze memory test.
Future experiments are aimed at further investigating the role of setd5 inactivation in the aging process, possibly revealing a link between the causes of intellectual disability and aging.

Keywords: Setd5, Zebrafish, ASD, ID, Aging

Title

Zebrafish as a model for sarcoglycanopathies: in-depth analysis of the disease phenotype

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Content

Keywords: sarcoglycanophaty, muscle diseases, zebrafish model

Sarcoglycanopathies (LGMDR3, LGMDR4, LGMDR5, and LGMDR6) are four severe autosomal recessive limb-girdle muscular dystrophies caused by mutations in the SGCA, SGCB, SGCG and SGCD genes resulting in the strong reduction of alpha, beta, gamma and delta- sarcoglycan (SG) proteins. The typical clinical phenotype includes progressive weakness of the proximal musculature of the pelvic and shoulder girdle, with variable cardiac and respiratory involvement.
SGs are single pass transmembrane proteins forming a tetrameric complex that localizes prevalently at the sarcolemma of striated muscle. The SG-complex is part of the major dystrophin-glycoprotein complex (DGC) and plays a key role in stabilizing sarcolemma during muscle contraction.
For the in vivo modelling of sarcoglycanopathies, different KI and KO mouse models have been generated so far, while in zebrafish only morpholino KD models have been produced.
Several are the advantages of using zebrafish as animal model, especially considering the similarity of the skeletal muscle structure and function with those of mammals. In addition, the preservation of several components of the DGC and sarcoglycan proteins (especially beta- and delta- SG) prompted us to focus on zebrafish for modelling LGMDR4 and LGMDR5.
We exploited the CRISPR/Cas9 technology to generate two KO mutants, the beta-SG-KO and delta-SG-KO. The genome editing system was adopted to target the initial region, exon 2, of the z-sgcb and z-sgcd genes where, thanks to the NHEJ, indel mutations were introduced. These mutations altered the reading frame of the genes, causing the formation of a stop codon few nucleotides downstream.
Despite the absence of a sarcoglycan subunit, the characterization of the two single KO mutants during the first 6dpf revealed a mild phenotype. We observed just small reduction in the embryo dimension, as well as a slight alteration in the organization of the skeletal muscle fibers. At resting conditions, KO embryos performed like wild type zebrafish and only if subjected to stressful conditions, it was possible to highlight a slight reduction in the swimming behavior.
By cross breading the beta-SG-KO with delta-SG-KO we obtained a double know out zebrafish, beta/delta-SG-DKO. In this case, a reduced dimension of the body length was clear evident already at 3dpf, as well as the presence of embryos with altered phenotype. The swimming ability was severely impaired as expected by the alteration of the skeletal muscle fibers. Considering the impact of sarcoglycanopathies on cardiac functions, we measured in the three models at 6dpf the heartbeats in one minute. It was interesting to observe that in the single KOs the heart seemed not affected by the absence of one sarcoglycan at this age. On the contrary, the beta/delta-SG-DKO embryos presented a decreased heartbeat that, together the previous describe features, highlighted the severe phenotype of the double SG-KO zebrafish line.
The histological characterization of the heart from adult delta-SG-KO line showed a thickening of the compact myocardium of the ventricle and a non-homogenous arrangement of the trabecular myocardium, suggesting the disease progressivity also in the single SG mutated zebrafish lines.

Title

Aluminium exposure affects the morphology of gills and muscles and routine oxygen consumption of zebrafish

Presenting Author

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Content

Aluminium (Al) is a metal highly diffuse in the Earth's crust. In the last decades, climate change and the associated phenomenon of acid rain are further increasing its environmental concentration. The acidification of water bodies facilitates the solubilization of the inorganic Al and increases its bioavailability for aquatic organisms. Studies carried out with different fish species have revealed that Al can produce toxic effects in fish by interfering with physiological activities and biochemical processes that, in turn, affect fertility, growth, and increase mortality. The neurobehavioral alterations link to the affections of swimming activity. However, until now, relatively scarce information is available about the effects of Al on the tissues relevant for the swimming performance of fish, such as gills and muscles. The gills of fish represent the first organ interacting with the metal in water bodies, and their alterations can affect the whole animal physiology. The impaired functionality of muscle is relevant for swimming behavior.
Here we report the effects of the chronic exposure to 11 mg/L of Al for 10, 15, and 20 days on gills and muscle morphology, in vivo routine oxygen consumption (rMO2, a measure of the whole animal resting energy requirement), and routine activity parameters (tail beats) of zebrafish adults. This experimental model is an optimal bioindicator to evaluate environmental pollutants' ecotoxicological effects.
The histological analysis highlights that the morphology of gills is altered by the Al treatment, although with different degrees depending on the exposure time. 10 days of Al exposure determines dramatic changes in branchial tissue which shows a disordered structure and a reduced lamellae number. After 15 days of Al exposure the branchial tissue shows signs of recovery, that is a more ordered structure and a higher number of lamellae. In 20 days- Al exposed animals there is a further morphological recovery. The morphological alterations of muscles parallel those of gills. A higher alteration of both white and red muscle tissues is evident in the organisms exposed for less time to Al, while a recovery of the normal morphological characteristics is observed after longer time of exposure. The recovery of muscle morphology is associated with its functionality, as demonstrated by the gradual increase of tail beats during the exposition. These changes reflect in the rMO2 consumption that is maximum after 10 days of exposure, suggesting a stress response for the animals. Then this parameter decreases and reaches the lowest levels after 20 days of treatment. This result suggests an adjustment of the resting metabolic pathways to face the reduced efficiency in the gaseous exchange of gill tissue.

Keywords: aluminum, routine oxygen consumption, gills, muscle, tail beats.

The electroencephalography of epm2a-/- zebrafish shows neuronal hyperexcitability distinctive of Lafora Disease

Presenting Author

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Content

Lafora disease (LD) is a rare fatal progressive myoclonic epilepsy characterized by drug-resistant seizures and neurological impairment. The condition is caused by mutations in the EPM2A gene encoding laforin, a dual-specificity phosphatase known to be involved in the regulation of glycogen metabolism. The main phenotypic feature of LD is the progression of seizures configuring a drug-resistant epilepsy that could lead to death after 10 years from the onset of the disease.
With the purpose of obtaining a new in vivo model, which could provide substantial advantages compared to murine model, we have firstly developed and characterized a novel epm2a-/- mutant zebrafish using CRISPR/Cas9 technology. Secondly, with the aim of assessing the potential role of autophagy in LD, we tested the early treatment of epm2a-/- larvae with Trehalose, an autophagy flux modulator.
Despite some variability, that can also occur in recording spontaneous seizures in patients with LD, at 120 hours post fertilization (hpf), the electroencephalographic (EEG) activity of epm2a-/- larvae showed significant neuronal hyperexcitability — with spontaneous recurring seizures — compared to wild-type larvae. Moreover, the early treatment of epm2a-/- 4hpf embryos with Trehalose — at a concentration of 150µM to the egg water — was able to significantly reduce the seizures-like events.
Our findings confirmed the important role that autophagy plays in LD and the Trehalose effectiveness in rescuing the epileptic phenotype, reducing the neuronal excitability and the frequency of spontaneous seizures. Furthermore, our results showed the reliability of zebrafish as valuable model of seizure-related disorders offering a rapid in vivo screening of potential therapeutic compounds. Even though further research in adult fish may be needed to amplify the significance of the present study, zebrafish larvae have proved to be the animal models of seizure-related disorders with the lowest capacity to experience pain or distress during EEG recordings complying with the EU directive on the protection of animals used for scientific purposes and with the “3Rs” principles.

Keywords: electroencephalography, epm2a, lafora disease, trehalose, zebrafish.

Title

Chronic Myeloid Leukemia transgenic fish: a new model for testing Tyrosine Kinase Inhibitors (TKIs) effects

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Content

Zebrafish has proven to be a versatile experimental model to study human hematopoiesis and it is a reliable in vivo tool for modeling hematological malignancies. Transgenic technology enables the generation of specific types of tumors by the expression of human oncogenes under tissue specific promoters and the vast majority of zebrafish leukemia models rely on overexpression of proto-oncogenes that are misregulated in human leukemias. Chronic myeloid leukemia (CML) is induced by the BCR-ABL1 oncogene derived from the t (9;22) translocation (Philadelphia chromosome). The BCR-ABL1 oncoprotein (P210) is a constitutively activated tyrosine kinase that persistently activates various signaling pathways regulating cell proliferation, transformation, thereby promoting leukemogenesis. To model CML, a transgenic zebrafish line expressing the human cDNA (BCR-ABL1) has been generated by using the Gal4/UAS system and then crossed with the HSP70-Gal4 transgenic line. A new transgenic line named bcr/abl-pUAS-CFPY//HSP70-Gal4 was obtained, hereafter Tg BCR-ABL1. In order to characterize the BCR-ABL1 expressing model, we deeply investigated zebrafish hematopoiesis and also tracked the molecular changes preceding the morphological phenotype as cell proliferation and inhibiting apoptosis by immunostaining for PH3, BrdU and acridine orange staining. Assays showed an increased number of proliferating cells and inhibition of apoptosis in Tg BCR-ABL1 fish. We analyzed different myelo-erythroid gene expression markers and Tg BCR-ABL1 showed over-expression of different markers, meaning a hematopoiesis reprogramming by BCR-ABL1 towards the myelopoiesis and granulopoiesis and less towards the erythropoiesis. Adult transgenic fish from 12 to 24 months showed a phenotype, similar to that in humans with an accelerated phase characterized by an increase in blasts and immature myeloid elements, and a blast phase with 70% blasts in both the peripheral blood and kidney marrow. Recent studies demonstrated that zebrafish shares 82% of disease-associated targets and several drug metabolism pathways with humans. The target therapeutic use of tyrosine kinase inhibitors (TKIs) of different generation, such as imatinib, dasatinib, nilotinib and bosutinib, has transformed the management of CML, largely turning a lethal disorder into a chronic condition. To determine if the pharmacological mechanism in Tg BCR-ABL1 transgenic was also conserved compared with CML patients, we treated the WT and Tg BCR-ABL1 embryos with the widely used anti-CML drugs, imatinib, dasatinib, and nilotinib (an optimal dose has been chosen after a dose-curve response) and with DMSO as a placebo. After incubation with these TKIs for 48 h, we evaluated the numbers of lcp1+ myeloid cells in WT and Tg larvae in the posterior blood island (PBI) region at 5 dpf. All the TKIs significantly reduced the number of lcp1+ myeloid cells in Tg BCR-ABL1 larvae compared with the DMSO control group. In addition, imatinib and nilotinib significantly reduced cell proliferation of myeloid population, inhibited the angiogenesis process and they showed a down-regulation of red cells in Tg BCR-ABL1 larvae. These preliminary results suggest that the pharmacological pathways in this model were similar to those in human CML. Understanding the underlying cause of resistance and screening for novel targeted drugs with low toxicity and high efficiency thus remain important steps in treating CML.

Poster Presentations

Title

Using zebrafish model for Alexander disease, a rare neurodegenerative disorder of the astrocytes

Presenting Author

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Content

Alexander disease is a rare but devasting brain disorder characterized by severe phenotypic manifestation such as seizures, intellectual disability, and developmental delay. Such disease is marked by defects in myelination and the appearance of astrocytes containing protein aggregates, also known as Rosenthal fibers. Heterozygous mutations in the glial fibrillary acid protein (GFAP) gene (1) have been identified in all patients and are responsible of the formation of Rosenthal fibers containing GFAP aggregates together with heat shock protein 27, alfaB-crystallin, ubiquitin and proteasome. At the present time, there is no cure for this disease and both in vitro and in vivo transgenic mice models of Alexander disease suffer from limitations in studying this disease. Zebrafish is commonly adopted in modelling human neurodegenerative diseases. The aim of this study has been the production of a transgenic zebrafish model for Alexander disease, based on Tol2 transposon approach and expressing the human GFAP gene carrying the most severe p.R239C mutation under the control of the zebrafish gfap gene promoter.
Then, we studied pTol2-GFAP WT-GFP zebrafish embryos with pTol- GFAP(R239C)-GFP by immunofluorescence and TEM, and we confirmed the presence of aggregates localized more frequently in glial cells expressing mutant than wild type GFAP. Then, we showed that both ceftriaxone treatments (2) and sHSPs stimulation positively affect mutant embryos p.R239C in terms of GFAP aggregates reduction (3). Then, we investigated the electrophysiological activity of neurons in transgenic zebrafish larvae by High-Density Microelectrode array platform. We observed a significant decrease in the head network burst duration and rate of GFAP R239C compared with control, compatible with the myelin disruption, typical of this disease. Overall, we propose zebrafish as a powerful model of Alexander disease, for both the study of the molecular pathogenesis and for high throughput drug screenings.

1.Brenner M. et al, Nat Genet 2001
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Title

Creation of a nsd1-mutant zebrafish line by CRISPR/Cas9 as a disease model for Sotos syndrome

Presenting Author

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Content

NSD1 (Nuclear receptor SET Domain-containing 1) gene maps on human chromosome 5 and encodes for a N-methyltransferase involved in the methylation of nucleosomal histone H3 lysine 36. In the chromatin organization, NSD1 works as a bifunctional transcriptional intermediary factor, depending on the complex cellular context. In humans, NSD1 genetic alterations are detected with high frequency in diagnosed cases of Sotos syndrome, an autosomal dominant overgrowth condition. This disease is characterized by prenatal and infantile overgrowth with advanced bone age, macrocephaly, facial dysmorphism, intellectual impairment as well as autistic traits.
In order to characterize the effects of NSD1 Loss of Function (LoF) in humans, the teleost Zebrafish (Danio rerio) results to be a useful disease model. In zebrafish, the NSD1 gene had an evolutionary duplication so we distinguish the two orthologues nsd1a and nsd1b, whose expression was characterized by real-time PCR during early developmental stages.
To generate a stable LoF mutant zebrafish line in the NSD1 orthologous genes, we microinjected zebrafish zigote with CRISPR/Cas9 ribonucleoprotein complex, initially targeting nsd1a gene. The efficacy of the mutagenesis has been verified in these mosaic embryos by high resolution melting analysis. After reaching the sexual maturity, these adult of F0 generation were outcrossed with wild type (WT) fishes to obtain F1 population. Indeed, the analysis on DNA extracted from F1 larvae allowed us to select the "founders", able to transmit one or more types of mutated nsd1a alleles to the offspring. Some of the transmitted indel mutations have been identified by Sanger sequencing and subsequently confirmed in F1 adults, which result to be heterozygous for the inherited mutation. Being patients with Sotos syndrome characterized by prenatal and infantile overgrowth, we conducted morphometric analyses of nsd1a mutants at 2 days post fertilization by comparing them with WT embryos. We also carried out a characterization of the craniofacial development of these mutants at 5 days post fertilization, using the Alcian Blue staining technique which allows the visualization of the cartilages, leading us to verify the presence of a possible phenotype of craniofacial malformations, reseambling a feature of individuals affected by Sotos syndrome. In addition, we are characterizing the heterozygous adult mutants in nsd1a gene performing behavioural tests, focusing our attention on a possible impairment of social interest that may resemble the autistic features of the patients. In addition, the creation of stable mutants in the other ortholog nsd1b is ongoing.
Meanwhile, we planned set up a protocol to modulate the levels of mRNA in the early stages of zebrafish embryonic development. To this purpose, we are employing the Cas13d protein, an endonuclease that targets RNA, using a specific RNA guide, determining its subsequent degradation. We synthesized and purified protein Cas13d and at the same time we obtained the cas13 mRNA by in vitro transcription using a plasmid as template. Initially we will perform proof of concept experiments, injecting the CRISPR/Cas13d system targeting tbxa mRNA that would generate a tailless phenotype. Then, the perspective would be to target the transcripts of nsd1a and nsd1b.

Title

Generation and characterization of the c19orf12 mutant zebrafish model

Presenting Author

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Content

Mitochondrial membrane Protein Associated Neurodegeneration (MPAN) is a rare neurologic disorder representing about 5% of all Neurodegeneration with Brain Iron Accumulation (NBIA) cases; it is caused by mutations in C19orf12 gene which encodes for a small protein found in mitochondria, endoplasmic reticulum, and at mitochondria-associated membranes. The available data suggest an involvement of the protein in lipid metabolism, mitochondrial function, and autophagy. Since in vivo models represent unique tools to study the pathomechanism in MPAN, we decided to generate a zebrafish model by a genome editing approach. The zebrafish genome contains four co-orthologues of the human C19orf12 gene: c19orf12a on chromosome 18 and c19orf12b1, b2 and b3 clustered in tandem on chromosome 7. c19orf12a protein product shares 59.9% of identity with the human protein and according to RNA-seq data is expressed at higher levels during the early stages of zebrafish development. For these reasons, we selected c19orf12a as the first target of our knock-out strategy. c19orf12a loss-of-function model was obtained using CRISPR/Cas9, delivered as ribonucleoprotein complexes by microinjection, performed at one-cell stage embryos.

The genomic sequence alterations were identified by Heteroduplex Mobility Assay (HMA) analysis and confirmed by Sanger Sequencing. A 2 bp deletion (Δ2) inducing a premature stop codon and an in-frame, potentially pathogenic, 3 bp deletion (Δ3) were selected and the fishes were outcrossed twice with wild-type fishes in order to obtain a pure second filial generation (F2), then incrossed to obtain pure homozygous mutant third generation (F3). The canonical Mendelian pattern of inheritance was confirmed by HMA.

By examining the mRNA using RT-PCR we observed that c19orf12a mutant transcript does not undergo nonsense-mediated decay nor is up-regulated as an attempt of a genetic compensation triggered by dysfunctional protein products. At the moment no antibody is available to analyse the protein product. We evaluated embryos phenotype from 24 to 120 hours post-fertilization, accurately monitoring head, tail and eye sizes and no significant differences were identified in comparison with wild-type embryos at the same stage.

To evaluate neuronal development, we studied the spatial and temporal expression of neuronal markers (neurod1, gfap, isl1a and dlx2a) by whole-mount in situ hybridization and we did not find any significant difference in the expression pattern compared to wild-type embryos. Surprisingly, the incross of Δ3-/- from the F3 generation was associated with embryonal lethality and all embryos died, within 21 dpf without showing gross morphological alterations. Despite the in-silico analysis indicating absence of off-target effects for the selected sgRNAs, we cannot exclude that we are facing artefacts. We are currently breeding a new F3 generation to further investigate this phenomenon. We could conclude that the absence of c19orf12 is not lethal, embryos development and nervous system morphology showed high similarity to controls. These preliminary findings suggest the necessity to evaluate also the co-othologues contribution in the mechanisms underpinning the development of neurodegeneration in MPAN.

Keywords: C19orf12 gene, MPAN, NBIA, neuronal development, zebrafish.

Title

Peripheral hypothyroidism in premature ovarian aging: an evolutionary study

Presenting Author

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Content

Thyroid hormones (THs) play pivotal roles in physiological processes, including aging. Their synthesis and secretion by the thyroid gland diminish during aging. Their balance is maintained by the hypothalamic-pituitary thyroid (HPT) axis and ensured by the local activity of enzymes (deiodinases), receptors and transporters.
Genetic and environmental factors, such as endocrine disruptors chemicals (EDCs), contribute to aging in different tissues. EDCs, including pesticides, can impair the ovarian health and promote premature ovarian aging altering the oocyte maturation as well as the activity of granulosa cells. Although debate, the alteration of circulating T3 levels have been associated with ovarian aging but the role of ovarian thyroid hormones metabolism and signaling is poorly characterized. The present work aimed to fulfil this gap characterizing the expression of deiodinases, TH transporters and TH receptors in an evolutionary study involving zebrafish and mouse females exposed to low-doses of chlorpyrifos (CPF) from early developmental stages to adulthood and women affected by premature ovarian aging.
In zebrafish, we evidenced a dose-dependent reduction of fertilized eggs in exposed females to CPF 30 nM and 300 nM. The premature ovarian aging was confirmed by the reduction of amh, gdf9 and bmp15 transcripts in females. The reduced fT3 levels and the pattern of expression of deiodinases (dio2, dio3a, dio3b) in the ovaries suggested the hypothyroidism of the organ, despite that none major change was observed in thyroid development. The reduction expression of igfbp1a mRNA, a T3 responsive gene, confirmed the ovarian hypothyroidism.
Although ovarian development, gamete maturation and the signaling pathways are conserved in vertebrate, zebrafish has asynchronous ovaries differently from mammals. Therefore, we confirmed the results in mouse females exposed to CPF 1 and 10 (mg/kg/die). Concordantly with the previous results, the exposure negatively impacted fertility in a dose-dependent manner and disarranged the levels of Amh, Gdf9 and Bmp15 mRNAs suggesting a premature failure of the granulosa cells and oocytes. Inhibition of ovarian T3 signaling was evidenced by the reduction of Spot14 mRNA. Similar results were obtained in a mouse model of human congenital hypothyroidism. These mice carrying heterozygous deletion of Pax8 (Pax8 +/-) or Nkx2.1 (Nkx2.1 +/-), or carrying both (DHTP), showed a reduced fertility. Although only the last ones were hypothyroid, we observed the disarrangement local of T3 (Dios, THRs, Spot14) signaling in infertile females.
The importance of an adequate T3 signaling in ovarian health and function was also confirmed in a study conducted in women with diminished ovarian reserve (DOR). In this last, all markers analyzed were impaired. As the matter of fact, fT3 levels were reduced in follicular fluid (FF) when compared to the CTRL group, the DIO2 mRNA detected in cumulus cells were dramatically reduced and the CPT1A mRNA was also reduced, confirmed the local hypothyroidism.
Overall, the data from evolutionary distant models underscore that the intra-ovary imbalance of T3 level might result in aging of the somatic cells and oocytes. Its impairment can be a common mechanism of action of genetic and environmental factors promoting premature ovarian aging and lifespan.

Keywords: Ovarian aging, Thyroid hormones signaling, Hypothyroidism, Evolutionary study

Poster Presentations

Title

A new transgenic reporter line reveals expression of protocadherin 9 at a cellular level within the zebrafish central nervous system

Presenting Author

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Content

The wiring of neuronal networks is far from understood. One outstanding question is how neurons of different types link up to form subnetworks within the greater context. Cadherins have been suggested to create an inclusion code where interconnected neurons express the same subtypes. Here, we have used a CRISPR/Cas9 knock-in approach to generate a transgenic zebrafish reporter line for protocadherin 9 (pcdh9), which is predominantly expressed within the central nervous system. Expression of eGFP was detected in subsets of neurons in the cerebellum, retina and spinal cord, in both larvae and juveniles. A closer characterization of the spinal locomotor network revealed that a portion of distinct classes of both excitatory and inhibitory interneurons, as well as motor neurons, expressed pcdh9. This transgenic line could thus be used to test the cadherin network hypothesis, through electrophysiological characterization of eGFP positive cells, to show if these are synaptically connected and form a discrete network within the spinal cord.

Poster Presentations

Title

Effects of three food dyes on motor activity in zebrafish embryos

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Content

Food dyes are widely used additives, and their harm to human health has been clearly recognized in recent decades. As a result, a significant effort has recently been made to introduce vegan substitutes prepared with fruit and vegetables. Although this category of dyes is widely used, not much information is available on their possible effects on aquatic and terrestrial ecosystems once released as waste into surface waters. The toxicity of artificial dyes on target organisms has been demonstrated. Our previous works highlighted the toxic effect of non-vegan food dyes on animal and plant model organisms [1]]. It is known that artificial food colors can induce hyperactivity and movement alterations [2,3]. To understand if vegan dyes can also have similar effects on locomotion, we exposed Danio rerio larvae to three common red food colors, a red vegan (VEG) preparation, the cochineal E120 and the artificial E124. We measured larvae motility and performed light and electron microscopy analyses of muscle. The larvae's average speed, acceleration, and length travelled significantly increase in the E124 treatments compared to the controls and the other two red dyes treatments. In semithin sections, muscles never show alterations in the morphology and/or in the general organization while metachromasia significantly decreases in connective septa exposed to E120 and E124. In E124 samples, TEM investigations also show asynchrony in fiber contraction, marked alteration in myofilaments organization and a significant vesiculation of mitochondria. In conclusion, our data highlight the marked toxicity of E124 for Danio rerio muscles and indicate that natural substitutions E120 and vegan red are not entirely harmless.

1. Motta et al. / Environmental Pollution (2019) 1126-1135
2. Doug et al. /Toxicology and Industrial Health (2013) 616-623
3 Eagle / Physiology and Behavior (2014) 174-179

Poster Presentations

Title

Functional validation in zebrafish models of candidate disease-genes and variants causative RASopathies and Golgipathies diseases

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Content

Keywords: rare diseases, neurodevelopment, disease-genes, RASopathies

Rare diseases represent a serious societal burden, with at least 70% of the cases manifested already during childhood in chronic forms often affecting the nervous system and resulting in extremely debilitating conditions. The lack of a fundamental understanding of the underlying pathophysiological mechanisms, which might involve a variety of cell populations and developmental processes, make them difficult to diagnose and treat. New and potentially pathogenic gene variants are continuously identified in undiagnosed patients thanks to advanced genomic technologies, resulting in an increased need for effective in vivo disease-models to obtain functional validation. In the framework of the “Undiagnosed Patients Program” at Ospedale Pediatrico Bambino Gesù (OPBG, Rome, Italy) more than 30 new rare diseases have been clinically and genetically classified since the launch in 2015. Here we show the most recent examples which benefited from functional validation through ad hoc modeling and analysis in zebrafish obtained at the newly established OPBG zebrafish laboratory. In this "in-house" in vivo workflow we utilized transient protein knockdown and overexpression approaches for loss and gain of function conditions, respectively, coupled to whole embryo imaging-based phenotype characterization at cellular and subcellular levels. We present zebrafish data which recently contributed to: 1. validate the pathogenicity of new disease genes and gene variants involved in novel nosological entities affecting neurodevelopment; 2. provide insights into the role of Golgi homeostasis and function for proper brain and body axis development (impaired in a new form of Golgipathy); 3. characterize the efficacy of newly synthetized molecules inhibiting the altered RAS/MAPK signaling involved in RASopathies.

Title

Antioxidant effects of conditioned medium from Wharton's jelly mesenchymal stem cells on developing zebrafish embryos.

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Content

Conditioned media harvested from stem cell culturing have the potential to be an innovative therapeutic tool against various diseases due to their high content of immunomodulatory, trophic and protective factors. Hence, the in vivo evaluation of the biosafety and efficacy of these products is essential. In this scenario, the zebrafish embryo provides an ideal platform for high-throughput toxicological analysis by allowing, at the same time, the minimization of the use of mammalian models without losing reliability.

In this study, we assessed the developmental effects provoked by exposure of zebrafish embryos to conditioned medium derived from Wharton's jelly mesenchymal stem cells.

Distinct batches of 6 hours-post-fertilization embryos were exposed to conditioned medium at dosages ranging from 5 to 350 micrograms/millilitres (referred to the total protein content of conditioned medium samples). In each assay, control groups of embryos from the same batches were exposed either to equivalent amounts of unconditioned medium or to a solution having the same inorganic salt composition and concentration as unconditioned medium. A further group of embryos was reared in embryo medium as a negative control.

Careful examination of control and treated embryos by daily stereomicroscope observation not only allowed the identification of the non-lethal and non-toxic conditioned medium dosage, but also revealed that exposure of embryos to unconditioned one in the same concentration range surprisingly inflicted major phenotypic deformities and provoked a dose-dependent increase in mortality. Acridine orange staining of live 72 hours-post-fertilization embryos revealed that the incidence of morphological aberrations in the vast majority unconditioned medium-treated specimens did correlate with the appearance of supernumerary apoptotic foci throughout the embryo body, including a relevant hotspot in the pericardial region. By contrast, conditioned medium-treated embryos did not change their spatial apoptotic pattern with respect to control unperturbed embryos, suggesting a favourable effect of conditioned medium against the onset of ectopic apoptosis. In strict accordance, real-time PCR analysis highlighted that the messenger RNA abundance of the two antiapoptotic markers bcl2-like 1 and delta113p53 was specifically increased, while that of the proapoptotic marker bim was drastically reduced, in conditioned medium-treated embryos.

Intriguingly, we also measured significant upregulation of sirtuin1, forkhead box O3a, catalase and superoxide dismutase 2 antioxidant gene expression in the same embryos. Chromatin immunoprecipitation analysis confirmed the permissive epigenetic state for catalase gene expression. Accordingly, colorimetric assays showed that the total antioxidant capacity of conditioned medium-treated embryos was remarkably higher than that of control embryos. Moreover, comparative observation of control and conditioned medium-treated 30 hours-post-fertilization embryos stained with the 2'-7'dichlorofluorescin diacetate fluorescent probe highlighted that the overall amount of Reactive Oxygen Species was almost completely abrogated following exposure to conditioned medium. Importantly, the staining also allowed to determine that exposure of fish embryos to conditioned medium is able to robustly alleviate the acute oxidative stress injury induced by hydrogen peroxide treatment.

Altogether, these findings lead us to conclude that exposure of fish embryos to conditioned medium confers protective effects against apoptosis and oxidative stress by upregulation of specific gene expression.