

Session 6: Emerging Issues

***In Vivo* Behavioral and Morphological Screening of a 1078-Chemical Library Using Zebrafish**

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Early developmental life stages are often uniquely sensitive to environmental insults, due in part to the enormous changes in cellular differentiation, proliferation, and migration required to form the required cell types, tissues, and organs. Molecular signaling underlies all of these processes. Thus, most toxic responses result from disruption of molecular signaling, making early developmental life stages the ideal time to determine if a chemical can perturb the expression or activity of essential molecular pathways. Our group's central goals are to identify bioactive compounds and to identify the molecular targets they act upon to disrupt vertebrate development. We developed an efficient screening process using embryonic zebrafish to assess chemical effects on behavior, morphology, and gene expression. As a proof of concept, we obtained the EPA phase I and II ToxCast chemicals, 1,078 compounds made up of pesticides, drugs, "green" chemicals, chemicals in cosmetics, and other consumer products. A static nonrenewal exposure paradigm was initiated beginning at 6 hours postfertilization (hpf) using a range of concentrations in 96-well plates. A total of 32 individual animals were assessed at each concentration. We also kept exposed embryos completely in the dark until 24 hpf and assessed a simple photo-motor response using the Photo-motor Response Assessment Tool (PRAT). PRAT quantifies individual embryonic photo-motor response following two pulses of bright light. The initial pulse normally results in pronounced movement, and the second light pulse usually produces no activity. At 120 hpf we also assessed photo-induced larval locomotor activity using Viewpoint ZebraBox to determine if chemical exposure impacted CNS-dependent motor responses. Finally, each larva was assessed for changes in a suite of 20 morphological endpoints at 120 hpf. We have successfully conducted the phenotypic and behavioral screening on all 1,078 compounds and a summary of the results will be discussed.

Cytochrome P450 in Fish

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Increasing use of small model fish in drug discovery and mechanistic toxicology demands knowledge of cytochrome P450 (CYP) gene regulation and function. CYP enzymes catalyze oxidative transformation leading to activation or inactivation of many endogenous and exogenous chemicals, with consequences in normal physiology and disease processes. Many CYPs potentially have roles in developmental specification, and many chemicals that cause developmental abnormalities are substrates for CYPs. We have identified and annotated the full suite of CYP genes in a number of fish genomes, including zebrafish, medaka, stickleback, killifish (*F. heteroclitus*), tilapia, and others, and compared these to the human and mouse CYP gene complement. We have also determined the expression of CYP genes during normal development in zebrafish. Zebrafish have a total of 94 CYP genes, distributed into 18 gene families found also in mammals. Other model fish species exhibit smaller, but similar gene numbers.

There are 32 zebrafish genes in CYP families 5 to 51, most of which are direct orthologs of human CYPs that are involved in endogenous functions including synthesis or inactivation of regulatory molecules. Across all fish investigated, the high degree of sequence similarity suggests conservation of enzyme activities for these CYPs, confirmed in reports for some steroidogenic enzymes (e.g. CYP19, aromatase; CYP11A, P450_{scc}; CYP17, steroid 17 α -hydroxylase), and the CYP26 retinoic acid hydroxylases. Complexity is much greater in gene families 1, 2, and 3, which include CYPs prominent in metabolism of drugs and pollutants, as well as of endogenous substrates. There are orthologous relationships of some CYP1s and some CYP3s between fish and human. In contrast, fish have many more CYP2 genes than the 16 found in human, with only two (CYP2R1 and CYP2U1) recognized as direct one-to-one orthologs based on sequence. Phylogenetic analysis as well as analysis of shared synteny identified CYP2 gene clusters evolutionarily related to mammalian CYP2s, as well as unique clusters in most fish species. Transcript profiling by microarray and quantitative PCR revealed that the majority of zebrafish CYP genes are expressed in embryos, with waves of expression of different sets of genes over the course of development. The results provide a foundation for the use of zebrafish as a model in toxicological, pharmacological, and chemical disease research.

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Zebrafish Models for Investigating Environmental Regulation of Adiposity

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The current epidemic of obesity and associated comorbidities constitutes a major public health challenge of our time. Obesity develops when excess energy harvested from dietary nutrients in the intestine is stored as fat in white adipose tissues. The rapid pace at which obesity rates have increased in recent decades suggests that obesity is determined in part by environmental factors. We have developed the zebrafish (*Danio rerio*) as a model system for investigating environmental and genetic factors contributing to adiposity and obesity. We have developed methods for high-resolution *in vivo* imaging of zebrafish adipose tissues, and used them to reveal that zebrafish and mammalian adipose tissues share extensive physiological, morphological, and molecular homologies. We also show that these methods are sufficiently robust for use in screens to identify novel factors involved in adipose tissue growth. We are currently using these methods to investigate two major types of environmental factors known to regulate adiposity: intestinal microbiota and pro-adipogenic environmental toxins. We have shown that intestinal microbiotas in zebrafish and mammals are dominated by the same major bacterial phyla, though there is little overlap at the genus and species levels. Strikingly, diet-dependent enrichment of bacteria from phylum *Firmicutes* occurs in the intestines of mammals as well as zebrafish, suggesting that ecological principles governing gut microbiota composition are at least partially conserved. Using gnotobiotic husbandry methods that we developed for the zebrafish system, we have shown that the presence of gut microbiota leads to increased harvest of dietary fats. We are now seeking to define the underlying mechanisms, and understand how these host–microbe interactions influence energy balance and fat storage in adipose tissues. We and others find that prominent environmental toxins, such as the organotin compound Tributyltin, can also promote adipogenesis. We are currently investigating potential interactions between Tributyltin and gut microbiota exposures on zebrafish adiposity. An improved understanding of the molecular mechanisms by which these environmental factors regulate adiposity should lead to novel strategies for reducing obesity and associated comorbidities.

Towards a Science-Driven Solution for Cooperative and Effective Management of Chemical Risks

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EU lawmakers have taken the bold and correct step of legislating the need to assess the toxicity of all chemicals sold in Europe in quantities of more than one ton per year (Registration, Evaluation, Authorisation, and Restriction of Chemicals [REACH]). This has spurred science to provide a robust and cost-effective solution. However, there are huge scientific constraints in determining the environment and human health risks for an ever-increasing number and diversity of consumer products, while toxicity testing fails to keep pace with modern biology. This has created an enormous backlog of chemicals that have yet to be assessed for potential health hazards.

Considering the scale of the current problem, and realistic future projections, a shift to next-generation assays is both timely and necessary. The proposed solution is to apply high-throughput toxicity testing with data-rich genomics assays to nonmammalian, ecological plus biomedical, model species (e.g. *Daphnia* plus *Drosophila*, *Hyallela* plus *C. elegans*, killifish plus zebrafish embryos), made reliable, inexpensive and efficient by laboratory automation.

Only by obtaining millions of standardized and quantitative genetic measurements will shared mechanisms of toxicity be tied to “susceptibility” or “adverse outcome” pathways and subsequently extrapolated to humans and used in understanding and managing risks. By contrast to alternative cell-based assays, a molecular database from toxicity testing on genetic panels of these selected whole-animal species that include well understood sentinels within natural ecosystems will facilitate across-evolution extrapolations that address both environment and human health issues.