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A new high-content model system for studies of gastrointestinal transit: the zebrafish

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Abstract

The zebrafish GI tract displays an anatomy and cellular architecture that is similar to the human GI tract, with concentric layers of inner epithelia, connective tissue, circular muscle, and outer longitudinal muscle layers. Propulsion of luminal content results from the integrated activity of smooth muscle cells, enteric neurons, and the interstitial cells of Cajal (ICC). Zebrafish larvae are transparent and propagating contractions in the entire GI tract are easily visualized. A new moderate-throughput zebrafish-based GI transit assay is described in this issue of *Neurogastroenterology and Motility*. This assay utilizes intact zebrafish larvae which contain essential regulatory elements (ICC and enteric neurons). Forward genetic analysis, which identifies genes underlying specific phenotypes, is possible using the zebrafish system. The zebrafish model system compliments existing models for studies of GI motility and will contribute to the understanding of the regulation of GI motility, and to identification of novel drug targets.

Introduction

GI motility results from complex and coordinated interactions between enteric neurons, interstitial cells of Cajal (ICC), and smooth muscles, and is also influenced by the central nervous system and the immune system. Regulation of GI transit is determined by dynamic changes in motility patterns. Motility patterns may be regulated from changes in single components, such as ICC, or alterations in multiple components. It is therefore essential to examine regulation of GI motility in intact and unperturbed physiological settings. Discovery of novel drug targets and the development of effective and safe drug treatments for GI dysmotility has proven challenging. A new model system that is tractable and that includes the relevant regulatory components of GI motility has high potential to facilitate target identification and validation for the treatment of GI disorders. Field et al describe a new method to quantify gastrointestinal (GI) transit in the zebrafish (*Danio rerio*) model system in this issue of *Neurogastroenterology and Motility* (4). The zebrafish model offers the important advantage for direct and continuous visualization of GI motility resulting from integrated actions of ICC, enteric neurons, and smooth muscle cells in an intact and unperturbed organism. Methods described by Field et al enable GI transit to be assayed in large numbers of larva. This is important because GI transit is variable between individuals and new drug treatments must be effective with this background. The zebrafish model also offers the exciting opportunity for identification of new motility regulatory genes because of

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the opportunity for forward genetic screens that may identify GI motility phenotypes. It is also possible to knock down individual gene expression using morpholino technology in embryos and larvae, although it should be noted that these effects diminish as the larva develops (2). The zebrafish has been a favorite model system for developmental biologists because development is rapid, and because developmental processes may be directly visualized. Zebrafish models for human disease are emerging rapidly and the ease with which organ function may be visualized is an advantage. This new model system that appears to be genetically and functionally similar to humans will compliment existing models and has the potential for the discovery of novel treatments for GI motility disorders.

Zebrafish oocytes are fertilized externally and the resulting embryos are transparent, allowing direct observation of organogenesis. Most organ development appears complete 3 or 4 days after fertilization (dpf) and larvae at this stage remain essentially transparent. However, full development of the external muscular layers of the GI tract occurs slightly later, between 5 and 7 dpf. It is possible to visualize organ function until 7 or 8 dpf, and treatment with 1-phenyl-2-thiourea inhibits pigmentation, allowing visualization of organ function at later developmental time points. This is appealing for the study of complex human diseases. In recent experiments tumor formation, angiogenesis, and metastasis in adult zebrafish have been directly visualized (25). This elegant in-vivo study is only possible using a transparent adult zebrafish model and it shows the dynamic process of tumor metastasis using human breast cancer cells in intact zebrafish. Cardiac function has also been modeled using the zebrafish. The 2 chambered zebrafish heart develops and is functional 24 hours after birth, and at 3 days post fertilization (dpf) drug response is highly similar to humans with respect to cardiac rhythmicity and atrial-ventricular coupling (15). Recent reviews describe zebrafish-based models for human cancers, cardiac function, immunology, schizophrenia, and obesity, and safety screens offered by small companies are also described (12, 15, 24), (1) (23),(11). Further evidence for the increasing popularity of this model is the increase in peer-reviewed publications and in NIH funded grant proposals (see figure 1) (14)

Validation of a zebrafish-based model system for human GI motility requires identification of the cellular elements that regulate and produce coordinated motility patterns resulting in effective GI transit. Development of a functional GI tract exhibiting spontaneous rhythmic muscular contractions is evident by 5 dpf, and this is also the approximate time that spontaneous feeding begins in the zebrafish. Longitudinal and circular muscle layers have developed, and enteric neurons populate the entire length of the GI tract at this stage (9, 17, 22, 26). Kit-like immunoreactivity first appears at 7 dpf, indicating the presence of ICC (20). These data are consistent with the emergence of highly coordinated motility patterns by 7 dpf (21). Coordinated motility patterns in humans require ICC, and loss of ICC is associated with motility disorders (3, 6). One recent study identified apoptotic ICC in human colon, supporting the concept that ICC density is dynamic, and results from the balance between apoptosis and proliferation (5).

Direct and continuous visualization of GI motility along the entire length of the GI tract is possible and contraction distance, velocity, and frequency may be measured using spatiotemporal mapping techniques (20). Holmberg and coworkers showed that TTX-

sensitive neuronal control mechanisms develop by 7 dpf (7). The essential regulatory elements, enteric neurons and ICC, and the effector cells, longitudinal and circular smooth muscles, are present and functional at 7 dpf. Motility patterns, measured from unperturbed, living zebrafish larvae, serve as a quantitative and functional readout of the integrated activity of each component. This high-content model system may contribute to a better understanding of the regulation of GI motility and enhance the identification, validation, and development of new drug targets.

A GI transit assay is validated in 6–8 dpf zebrafish larvae by Field et al (4). This straightforward assay is possible because larvae spontaneously ingest powdered food labeled with a fluorescent tracer. Tracer movement is directly imaged at specific time points during the subsequent 24 hour period. The authors clearly demonstrate one experimental advantage of the zebrafish model, using 1416 larvae to quantify intestinal transit in 7 dpf larvae! This large number of experiments shows clearly that larvae may be functionally divided into a fast transit group, when all tracer is passed within 6 hours, or a slow transit group, when it takes 24 hours or more to pass tracer. Close examination of slow transit larvae shows that 40% of the slow transit larvae have tracer remaining in the GI tract at 24 hours. The authors validate the assay by blocking development of enteric neurons using morpholino knockdown of *ret* expression, resulting in a decrease in intestinal transit, as would be expected with the absence of the essential neural regulatory component for GI motility. The authors also provide essential details regarding feeding behavior; larvae do not eat in the dark, and only ~50% of the larvae spontaneously ingest dry food labeled with marker. Transit appears to pause between 6 and 12 hours and the authors suggest this may result from light/dark cycles or circadian rhythms. It will be interesting to learn the physiological factors that regulate this delay.

Mouse models with defective Kit signaling have correlated reduced ICC density with abnormal GI motility patterns, and have identified cellular factors that influence ICC density (18). Motility is uncoordinated in *W/W^v* and the *Sl^d* (*Steel-Dickie*) mouse mutants which lack tyrosine kinase activity because the Kit receptor has reduced function or the stimulating ligand is unavailable (18). It is reasonable to examine kit mutants in the zebrafish to ascertain zebrafish kit function, but comparing zebrafish and mouse models is complicated because the zebrafish expresses co-orthologous pairs for Kit (*kita* and *kitb*), and also for stem cell factor (*kitla* and *kitlb*) (8, 13). These 4 genes are expressed in the adult GI tract (20). Sequence comparisons show that *kita* and *kitb* contain putative tyrosine kinase domains which are highly similar to human *Kit*, and extracellular domains which are less similar (13). One zebrafish Kit mutant exists, *Sparse*, a null *kita* mutant with defects in skin pigment patterns (19). Contraction frequency of GI muscles is reduced and the area of the gut is expanded, a phenotype that is similar to *W/W^v* and the *steel* mutants (20). Recent experiments in our lab have shown that *sparse* develops coordinated motility patterns more slowly and that Kit-positive cells are present in adult *sparse* GI tissues suggesting the *kitb* is sufficient for ICC development and maintenance (unpublished observations). The role for *kitla* and *kitlb* on Kit signaling in the zebrafish has not been examined. Sequence comparisons show that *kitla* appears similar to soluble stem cell factor whereas *kitlb* appears more similar to membrane-bound stem cell factor (8).

The zebrafish contains duplicate copies for many human genes. Differences in gene copies may indicate separate, but essential functions for each copy. Retention of co-orthologous pairs therefore suggests separate functions for the 2 forms of the Kit receptor and for each Kit ligand. It is possible to examine the specific roles for membrane-bound and soluble stem cell factor on ICC development, maintenance, and ultimately on ICC plasticity using the zebrafish model.

The zebrafish GI tract contains the regulatory elements necessary for the generation of coordinated motility patterns, and this new GI transit assay supplied by Field and coworkers offers a medium throughput assay for GI transit (4). Spatiotemporal mapping techniques provide important details about GI motility patterns in zebrafish larvae (7) (21). This new model system and this assay will complement existing models examining the regulation of GI motility. However, it offers some unique benefits including the opportunity to visualize motility patterns in unperturbed organisms, the opportunity to use a forward genetic screen to identify novel regulatory genes, and reverse genetic screens to determine the specific role of selected genes on GI development. Methodological tools provided by Field et al will contribute to future studies to correlate development and regulation of zebrafish GI motility patterns with human GI motility, and to define the specific regulatory components in the zebrafish. When these are fully realized, our understanding of the genetic and gene-environment interactions that control GI motility will be enhanced. Finally, the Zebrafish system offers opportunities for drug development because medium-throughput screen for GI transit in an intact organism is now possible. Only small quantities of compounds will be needed to examine motility effects, and visualization of motility patterns is possible.

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Figure 1. NIH funding of new grants zebrafish-based research has increased rapidly. Data were obtained from a CRISP database search using keywords zebrafish and danio, and requiring grant numbers to contain "01".