REPORT AND RECOMMENDATIONS TO DEVELOP THE ZEBRAFISH SYSTEM FOR GENETIC STUDIES OF VERTEBRATE EMBRYOGENESIS AND DISEASE

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Status of the Field and Recommendations

A meeting entitled "Current Advances in Defining the Zebrafish Genome" was recently held at Children's Hospital, Boston. The meeting brought together an international group of investigators who focus on the study of zebrafish. A consensus view of the community is elaborated in this report.

The premise: Mutagenesis is a powerful way to identify genes necessary for any specific biological function. In zebrafish, robust methodologies permit the efficient generation and recovery of mutations in genes that regulate developmental patterning, organ morphogenesis, cell and organ physiology, and animal behavior. Evidence shows that the functions of many of these genes are conserved among various groups of vertebrates. Thus, an analysis of zebrafish mutations provides a unique opportunity to understand gene functions shared by all vertebrates, including humans. Zebrafish mutations thereby promise to aid the transition of the human genome project from descriptive to functional genomics.

A. Status

1. *The zebrafish as a developmental system.* Zebrafish embryos develop externally and are optically clear, making embryogenesis and organ development directly visible in living embryos. The development of normal embryos and larvae has been extensively characterized. Gene expression at the single cell level is easily studied in zebrafish. The readily accessible embryos facilitate cell transplantation experiments, thus allowing exquisite characterization of cell fate decisions.

2. *Genetics in the zebrafish.* Because of their relatively short reproductive cycle, the ability to house large numbers of animals in a small space, and the large number of eggs produced by each female, the zebrafish is the most efficient vertebrate model system yet utilized for collecting a large number of embryonic lethal mutations at anything approaching saturation mutagenesis**.** Investigators

at the University of Oregon have created many mutations of the zebrafish, and recently two large scale screens done at Max-Planck-Institut, Tübingen, Germany and at Massachusetts General Hospital, Boston have produced over 2000 independent mutations in about 500 genes essential for embryonic development**.** These mutations cause defects in early embryonic patterning or they derail the development of various organ systems. Several mutants have disorders similar to human disease states. The genetic and phenotypic characterization of these mutations and the cloning of the defective genes should provide tremendous insight into the molecular genetic mechanisms that drive normal development and organogenesis in vertebrates.

3. *Current state of zebrafish genomics.* NIH support has been critical for the birth of zebrafish genomics research: the NCRR has funded the isolation of the 1200 currently available microsatellite, RAPD, and cloned gene markers. Together, these markers provide a genetic linkage map with a 2.5 cM resolution on average. All centromeres have been localized on the linkage map, and the map is closed (newly mapped loci fall onto one of the 25 linkage groups which represent the 25 chromosomes). While the number of markers is insufficient for isolation of the defective genes by standard positional cloning approaches**,** uncharacterized RAPDs and / or AFLPs closely linked to mutations can be obtained by bulked segregant analysis, but these techniques are labor intensive. Large insert libraries (YAC, PAC, and BAC) have been generated for chromosomal walking. No physical map exists. Zebrafish-mammalian radiation hybrid and somatic cell hybrid panels have been established for the rapid positioning of candidate genes on the genetic map. The availability of haploid and gynogenetically diploid zebrafish allows rapid positioning of mutant genes on a framework linkage map. After the localization of a mutation, candidate cloning approaches are easily utilized in the zebrafish because the large clutch size permits families in excess of 3000 embryos, thereby allowing the construction of fine structure genetic maps in the realm of a few tens of kilobases resolution. Mutations in known genes have been identified for about nine complementation groups by the candidate gene approach. There is a high level of conservation of synteny between human and zebrafish chromosomal regions, almost as much as between mouse and human. A key premise driving zebrafish research is that relational databases between mammalian and fish genomes will permit the zebrafish genome to function as a mutational map for mammalian genes.

4. *Insertional mutagenesis.* A method using retroviral vectors has recently been described. Although lower in efficiency than chemical mutagenesis, insertional mutagenesis has already resulted in the cloning of four vertebrate genes whose function had not previously been known, and each is associated with a specific mutant phenotype.

5. *Availability of mutants.* The mutants are currently available through the large centers at the University of Oregon, Massachusetts General Hospital, and The Max-Planck-Institut, Tübingen. Currently, funding is not available for the maintenance of the stocks or the distribution of the mutants to individual laboratories.

6. *Need for comprehensive infrastructure.* It is currently possible to isolate a novel gene in the zebrafish; however, a substantial effort is required. Studies of the zebrafish genome have been rapidly developing, but the genomics is not up to date with studies of other species. The zebrafish requires additional infrastructure to ensure success as the community enlarges and embraces the medical establishment. There is a need to interact with other genome communities. The study of the zebrafish genome and the interfacing with other species such as pufferfish, mouse, and human, should allow a complete analysis of vertebrate genome**s**.

B. Recommendations

The director of the NIH charged us with developing a plan for infrastructure in three areas: genetic resource repositories; informatics; and genomics.

1. *Regarding repository issues.* A center should be established to maintain irreplaceable mutant zebrafish stocks. A Zebrafish Stock Center will provide frozen sperm samples, heterozygous fish, and embryos to investigators upon request. The center should be closely associated with a research group to aid in the training required to identify the various mutations. The cost of maintaining mutant fish stocks, as well as providing these stocks to investigators is expensive and currently unsupported. The community of zebrafish researchers who met in at the Cold Spring Harbor Laboratory meeting in 1994, recognized the importance of a centralized stock center and appointed a committee to work towards establishing a center. Since then, Christiane Nüsslein-Volhard has converted a small part of her laboratory in Tübingen into a stock center, currently maintaining fewer than 100 of the mutant lines from her own laboratory, but it is unclear how mutant embryos or adult heterozygotes can be shipped to investigators in the United States or whether this center will be able to expand to meet the needs of the entire zebrafish research community. In the US, Wolfgang Driever and Mark Fishman initially tried to establish a stock center in Boston; however this plan was abandoned in 1996 when it became clear that there was inadequate support from local Universities and that construction and operation of a Center would be prohibitively expensive in a major metropolitan area. The current community plan is to establish the stock center at the University of Oregon. A grant proposal with Monte Westerfield as a supervisor has been submitted to the NCRR at the NIH; however the funding for this remains to be determined. We recommend that a main United States

Stock Center be established in Oregon and that support be directed to additional centers such as Tübingen.

2. *Regarding informatics.* The zebrafish community currently maintains a Web Page supported by the University of Oregon. This site contains a web-based version of the Zebrafish Book edited by Monte Westerfield, rules for naming mutations, addresses of members of the zebrafish community, a bibliography of zebrafish, and links to other zebrafish sites. A program funded by a starter grant from the NSF and the Keck Foundation with zebrafish biologists and computer scientists as co-PIs aims to provide access to a relational database containing a morphological staging series, phenotypic descriptions of mutant lines, photographs and descriptions of gene expression data, and an interactive mapping protocol. We recommend that an advisory panel be set up that includes experts from the other genome communities to discuss the informatics needs of the zebrafish community, and that funding become available for implementing these programs.

3. *Regarding genomics.* The molecular and functional analysis of zebrafish mutations would be tremendously enhanced by the following items:

1) 100,000 expressed sequence tags (EST's). These tags would provide genetic markers, suggest candidate genes for zebrafish mutations, help identify orthologous genes among vertebrates, and characterize the extent of conserved syntenic regions between zebrafish and humans. An EST project should include sequences of both 5' and 3' ends of the clones. The 5' sequences provide gene structure information that is useful for identifying orthologues among vertebrates, while the 3' ends provide unique sequences useful for genetic mapping. The 3' ends also provide gene-specific probes for expression studies.

2) A radiation hybrid panel has been created and is currently being grown up and aliquoted. Over 10,000 ESTs should be placed on the radiation hybrid panel using the sequences of the 3' untranslated regions. Comparative mapping with human and mouse genomes should be a priority to identify the borders of conserved chromosome segments.

3) An effort should be generated to establish a 1 cM microsatellite map. Genes and markers already on the existing maps should also be scored on the radiation hybrid panel.

4) A framework physical map should be established, probably by fingerprinting the existing zebrafish BAC and PAC libraries. The physical map should be tied to the genetic map establishing which PAC and BAC clones contain which mapped genetic markers.

5) The establishment of a centralized system for cDNA mapping would allow high throughput analysis of the genome.

4. *Insertional Screens.* A large scale insertional mutagenesis screen would complement genome efforts such as cloning of mutations. It is established that

such a screen could be done by two to three laboratories and would generate mutations in a substantial fraction, perhaps 1/3 to 1/2, the embryonic essential genes within a three year time period. These insertions could be provided to the community for rapid characterization and isolation of interesting mutant genes.

5. *Other fish-specific technology*. It is possible that specific technology will be developed in the future for isolating fish genes. An effort should be made to fund fish-specific technology that advances the general field of genomics. By funding projects such as locus-trap technology, it is possible to develop the fish as a model for future genome efforts. Other technologies which should be funded is the generation of zebrafish ES cells coupled with efficient gene targeting, and the generation of stable transgenic lines with green fluorescent protein (GFP) targeted to various tissues.

C. General Comments

1. *Continued re-evaluation.* Every one to two years, a meeting should occur to discuss the efforts in the fish community to implement these recommendations.

2. *Evaluation of zebrafish in the study of vertebrate disease states.* Funding should be targeted to encourage studies on zebrafish that are relevant to disease in humans or other mammals. This includes zebrafish mutations that represent human diseases as well as the specific screens which target processes that affect disease states. An R01 or RFA format should be utilized for these proposals.

REPORT AND RECOMMENDATIONS FOR THE ZEBRAFISH SYSTEM

Introduction

Vertebrate development has been characterized extensively by studies of classical embryology, molecular biology and biochemistry. Mutational analysis among vertebrates, however, has lagged behind such investigations in invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans.* Despite the difficulties of mutagenesis in vertebrates, many important mutations blocking key steps of embryonic development have been isolated and studied in the mouse and to a lesser extent in humans. Recently, a mutational approach to understanding organogenesis and early embryonic patterning has been undertaken in the zebrafish. The zebrafish embryo is optically clear and can be screened for developmental mutations under a light microscope. Two large scale screens for developmental mutants have been performed. Male spermatagonia were treated with ethylnitrosourea, a chemical known to cause point mutations. These fish were mated to females, and F1 and F2 families were generated. After intercross mating of the F2 families, the embryos were screened under a light microscope from day one to five for defects in embryogenesis.

Over 2000 individual mutations were generated with specific defects affecting early embryonic patterning or organ development. Mutations giving similar phenotypes were tested for complementation and define 500 complementation groups. The mutations affect the establishment of body axes, the cell cycle, the morphogenesis of organs, and the biological function of organ systems, as well as specific biochemical pathways. Specific screens are additionally being done to examine the function of distinct organs and define more mutants in other developmental pathways. It is likely that many novel genes are affected in these mutations. To date, only nine of the 500 genes have been cloned.

The genome effort in the fish community has rapidly accelerated over the past three years. Currently, there is a map which consists of about 500 microsatellites**,** 600 RAPDs, and more than 125 genes. This allows positioning of genes with at a resolution of 2.5 cM. The 25 chromosomes of the zebrafish have recently been individually identified by replication banding and a standard karyotype has been established, but only one linkage group has as yet been assigned to a cytogentically identified chromosome. Large insert libraries exist including BAC, PAC and YAC libraries, and both somatic cell and radiation hybrid panels have been generated. SSCP and RFLP mapping has positioned over 125 genes on the working map. Thus, reagents exist that are necessary for positional or candidate cloning in the zebrafish.

During the development of this proposal, we considered the following three issues:

1) What can the analysis of zebrafish development and physiology contribute uniquely to further our understanding of biology and pathology shared among all vertebrates, including humans?

2) What infrastructure is needed to support the unique contributions of zebrafish?

3) How could the NIH facilitate the development of the general infrastructure of the zebrafish community to allow rapid isolation of these genes?

Rationale for the Zebrafish Genome Effort

Mutagenesis is one of the best methods for identifying gene function. Of all vertebrates studied to-date, mutations can be made and recovered most readily in the zebrafish. The availability of novel mutations that disrupt zebrafish development should allow the isolation of interesting genes relevant to the study of human development and disease. More specifically, a correlation to the human genome should allow the zebrafish to be an indicator of human gene function. It is critical to develop the zebrafish system for rapid isolation of

individual mutant genes, and to expand informatics to allow human genome efforts to utilize knowledge of zebrafish gene functions**.**

Tremendous Growth in Zebrafish Research

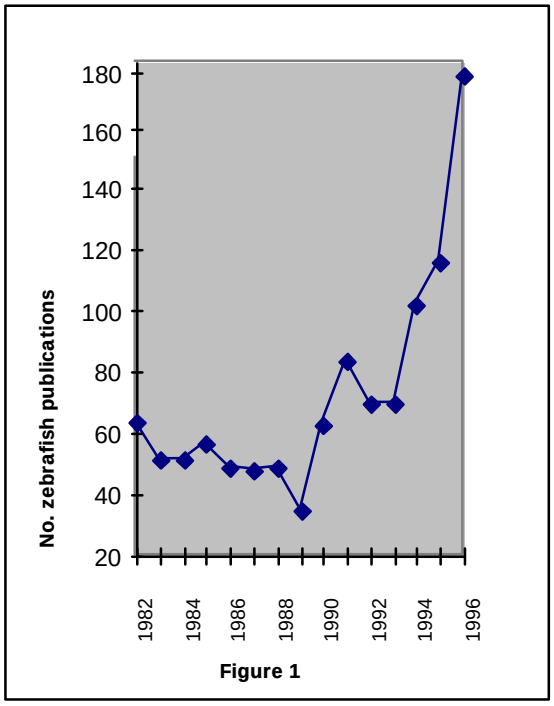

Figure 1

The first paper describing developmental zebrafish mutants was published in 1988 (Grunwald et al., 1988). Since then, there has been an explosion of interest (Travis, 1996) and research in this species (Fig. 1). Many new laboratories began projects on zebrafish development and genetics. In 1990, the National Institutes of Child Health and Human Development sponsored a small meeting of about 35 invited participants in Oregon that explored the potentials of zebrafish as a system for studying developmental genetics (Barinaga, 1990), and similar meetings were held in England and Germany a year later. A newsletter was started in 1991; it is currently in its 15th issue with a mailing list of over 700 researchers. The practical lab manual for zebrafish is now in its third edition (Westerfield, 1995) and has sold over 2,000 copies. Practical courses on the laboratory use of the zebrafish have been taught in Japan, England, Germany, and the US. The first open-invitation meeting was held at Cold Spring Harbor in 1994 with a second meeting in 1996. Since the first meeting, the mailing list has more than doubled and the number of labs studying zebrafish continues to grow at an increasing pace. Screens for developmental mutants are now ongoing in at least 8 separate cities (Driever et al., 1994; Mullins et al., 1994) and many thousands of new mutations have been isolated. The number of new labs working on zebrafish continues to grow; over a dozen postdocs from the University of Oregon labs alone have started new labs in the past two years.

SPECIFIC ISSUES:

I. Repository Issues

In addition to the well-publicized mutational screens, smaller labs have been generating mutations throughout the world. Fish carrying mutations are now distributed among more than 100 laboratories in 28 countries. The zebrafish research community desperately needs a centralized site to preserve and keep track of this genetic resource, and to facilitate their distribution to researchers.

Recommendations

A grant has recently been submitted to NIH (NCRR) to fund a National Resource for Zebrafish. The P.I.s of this grant include a zebrafish embryologist (Westerfield), one of the leaders of the recent large screens (Driever), and a fish pathologist (Mike Kent). They propose to establish a stock center to maintain wild-type and mutant stocks of the highest possible quality and uniformity and to make these stocks readily available to the international research community.

The proposed stock center will distribute not only fish, but also information. It will:

1) develop and maintain a computer database, accessible via the Internet,
2) publish a manual for the laboratory use of zebrafish and a periodical with news about zebrafish research and techniques,
3) facilitate communication among zebrafish researchers, and
4) host visits from researchers who wish to work with stocks or learn techniques to identify and maintain mutants.
5) The stock center will also develop methods to improve the health of laboratory zebrafish. It will:

    a) establish standards and procedures for generating healthier and more vigorous strains,
    B) characterize diseases endemic to laboratory stocks,
    C) develop methods for disease control and treatment and
    D) publish a manual of procedures for preventing, diagnosing, and treating zebrafish diseases.

The University of Oregon has identified a building which can be renovated to hold the stock center. The University recognizes that success of the stock center is crucial for efficient and widespread study of zebrafish mutations and is, thus, committed to the establishment of this center. The University is willing to act as the general contractor for renovation and will provide University services at no cost.

II. Informatics

The remarkable success of research using zebrafish has generated a serious information access problem. Although the use of zebrafish in genetic research is relatively new, the number of labs and the amount of data generated by these labs are increasing at a rapid rate. For example, ongoing genetic screens have already identified over 2,000 mutations and we anticipate that this number will increase several fold in the next few years. As mutant lines become available, the information about these lines grows rapidly; studies of each mutant generate a description of its phenotype, genetics, inheritance, map location, and interactions with other genes. The number of annual zebrafish publications has increased over 400% in the past six years. The information already far exceeds the ability of individual scientists to track and organize it. For these reasons, a systematic and concerted effort has begun to establish and maintain a centralized database for the zebrafish community.

A group of zebrafish researchers was appointed at the 1994 Cold Spring Harbor meeting on Zebrafish Genetics and Development, to establish an on-line database of information. The Informatics Project is currently funded by a starter grant from the NSF (BIR-9507401) and a grant from the W.M. Keck Foundation. The database is being designed by a collaborative effort of biologists (Monte Westerfield, Univ. of Oregon, and Wolfgang Driever, Univ. of Freiburg, Germany) and computer scientists (Sarah Douglas, Ekehart Doerry, University of Oregon). Representatives from the Mouse Gene Expression Information Resource (Martin Ringwald, Jackson Lab; Johnathan Bard, University of Edinburgh) and the Mouse Genome Database (Janan Eppig, Jackson Lab) serve as consultants to facilitate comparisons using zebrafish and mammalian databases. There are mirror sites in Strasbourg, France, and Tokyo, Japan, and an additional site is being established in Germany. Design of the zebrafish database is being coordinated with similar efforts in other organisms; the database developers are participating in gene expression database, nomenclature, and informatics workshops this year.

The purpose of the database is to provide public access to developmental and genetic/genome data from the zebrafish. The database is accessible through a graphical interface to the WWW (http://zfish.uoregon.edu). Users can run common types of searches directly from the WWW interface; more unusual queries can be run through the system administrator.

The database is currently in a "beta-testing" phase in which a dozen scientists from around the world with authorized access are submitting and searching for data. Feedback from this testing phase is used for further design and refinement of the database. Public release of parts of the database is scheduled for later this year. Some of the data are entered by "authorized users" whereas others are entered by

the system administrator. To ensure all information (even if incomplete) is retained by the community, unpublished as well as published data will be included; published information will be marked so that users can assess the confidence level of each entry. The database contains text, image and graphical data. Anatomical data, like gene expression patterns, can be searched spatially, based on graphical representations of the embryo. Initial estimates are for the database to contain approximately 6000 images documenting the expression of 150 genes. In addition, the database will have an atlas for staging embryos, an anatomical atlas of the embryo, information on approximately 5,000 mutants (including images), as well as genetic information (gene names, markers, genetic map), information on probes, antibodies, etc., a bibliography, and lists of people working on the zebrafish.

Recommendations

The NSF starter grant will expire in August of 1997. By that time, initial development of the database system and graphical interface should be completed. However, the bulk of the data will still need to be entered and as new data become available through future studies, ongoing support will be required to maintain and expand the database. A long term goal of the database project is to facilitate conserved syntenic genome analysis and comparisons of gene expression patterns among various organisms. Thus, funds will be required to allow coordinated development of the zebrafish database with genomic and gene expression databases from other organisms.

III. Genomics

The recent meeting in Boston focused most of its efforts on understanding the status of genomics in the zebrafish. The following is a report of the meeting.

Mapping Mutant Genes

The isolation of mutant genes is facilitated by the prior existence of a map of the zebrafish genome, which includes RAPDs (random amplified polymorphic DNA) and CA repeats. The RAPD map was assembled by John Postlethwait and coworkers at U. Oregon and is composed of 652 RAPD markers. 100 genes have been mapped to 25 chromosomes. The present CA repeat map was created by Howard Jacob and Mark Fishman at Mass General Hospital and consists of 500 microsatellite markers. Together, the map has a resolution of 2.5 cM. Mark Fishman described the CA repeat map and his goal is to increase the number of microsattelite markers to allow a resolution of 2 cM. This will facilitate both candidate and position cloning approaches in zebrafish.

Mutations can be mapped by identifying genetic markers that segregate with mutant phenotype. These markers can be RAPDs, CA repeats or cDNAs. The

markers are defined as polymorphic differences between the strain on which the mutations were created (AB or Tu) and polymorphic strains (DAR, SJD, WIC and INDIA). Several mapping techniques are currently available. In the first method, the eggs of a hybrid (AB/dar) female heterozygous for the mutation of interest are fertilized with UV irradiated sperm to create haploid embryos. Haploids develop normally until day 4 and many mutant phenotypes can be scored in these animals. For a heterozygous female 50% of the haploid embryos will be wild-type and 50% will be mutant. Polymorphic markers that are linked to a mutation can be identified by looking for the segregation of markers between pools of wild-type and mutant embryo DNAs. This technique is termed bulk segregant analysis. A similar analysis can be done using wild-type and mutant embryos from a diploid cross between two heterozygous hybrids. A third technique, half tetrad analysis, is very useful for rapidly placing a mutation on a linkage group. In zebrafish, it is possible to create gynogenetic diploid embryos by applying pressure to eggs fertilized by UV irradiated sperm. This pressure blocks meiosis II, resulting in the production of diploid embryos. These embryos are homozygous for all loci proximal to the first cross-over on all chromosome arms. Therefore, embryos that are homozygous for the recessive mutation will almost always be homozygous for the allele of the centromeric marker linked in coupling to the mutant allele in the parent. One can screen for centromeric polymorphic markers (RAPDs, CA repeats or cDNA primers) that segregate with the mutant phenotype. Once the linkage group is determined one can use the currently available maps to more precisely define the map position of a mutant.

Using the current maps of the zebrafish genome, the closest linked marker would be on average 2.5 cM from a mutation; however, in order to start position cloning a marker 1 cM from the mutation is generally required. In cases in which a 1 cM marker is not available, two techniques can be used to isolate closer markers. The first technique uses RAPD primers. There are over 2000 RAPD primers that have been developed at various labs, mostly as a reagent for plant molecular genetics. Bulk segregant analysis of haploid embryos with these primers will test over 6000 loci, which should produce markers 1 cM away from a mutation. A newer technique, Amplified Fragment Length Polymorphism (AFLP), can also be used to screen the genome for tightly linked markers.

Large Insert Libraries

Several investigators described the construction of zebrafish YAC and PAC libraries. The PAC, a YAC, as well as a BAC library are available from Genome Systems. Another YAC library should be available from Research Genetics very shortly. The YAC libraries have inserts of anywhere from 300 kb to 1 megabase and the PAC and BAC libraries average between 80 to 150 kb inserts. Each library represents at least 5-6X coverage and has been probed by multiple laboratories, suggesting that the libraries are fine for positional types of approaches. At the meeting, Alex Shier and Will Talbot described their use of these libraries to walk

through the one-eyed pinhead locus. They have a complete BAC contig through the region and are in the process of making cosmid libraries from the BAC to isolate their mutant gene.

Once a BAC contig is arrayed through the locus, several techniques are possible for positional cloning and isolation of the affected gene. These include standard techniques such as exon trapping and cDNA selection. In situ hybridization studies could be done to search for expressed exons relevant to a mutant phenotype, i.e. expressed at the right place and the right time to cause the defect. Another possibility is to sequence the entire BAC clone itself. This is a costly approach, but will ensure the isolation of the exonic sequences. Phenotypic rescue, reminiscent of cosmid injection in worms, could also be tried. Genomic clones (PAC, BAC or cosmid) could be injected into zebrafish embryos in an attempt to rescue the phenotype. Such a rescue has been demonstrated for the *floating head* mutation. This approach, which could not be done in many other vertebrate species, will also allow the rapid isolation of genes.

Candidate Cloning

Currently over 100 genes have been mapped on the zebrafish genome using RFLP or SSCP analyses. The location of 100 cDNAs predict that two duplications occurring in vertebrate evolution prior to the development of teleosts and also demonstrate the conserved synteny between vertebrates. There are many large chromosomal segments that are conserved between humans and zebrafish and it is possible that the level of synteny between mouse and humans is equivalent to that of zebrafish and humans. Given the EST efforts in human and mouse, it may be possible to map mutations in the zebrafish, and to establish candidates for these mutations based on relative syntenic map position between humans and mice. cDNAs are traditionally mapped by isolating the 3' UTR sequence and defining a polymorphism between polymorphic strains. The map position is determined by following the segregation of polymorphisms in the currently available mapping panels. Over nine mutations have been studied in this manner with candidate clones directly assigned to a mutation by defining a polymorphism and showing absolute linkage to the mutant phenotype. The mutant allele is then sequenced and the mutation is defined. This approach is extremely rapid and suggests that one goal of the zebrafish community should be to map more candidate genes.

Size of the Zebrafish Genome

There was considerable controversy about the actual size of the zebrafish genome, which may be one third to one half that of humans. The genome size was originally estimated in the 1960s by measuring the DNA content of erythrocytes. This needs to be repeated, because a precise measurement is necessary in order to calculate the physical size relative to the genetic size of the zebrafish genome. The

present estimate is that 1 cM represents 600 kb, but the distance could be even smaller, perhaps 200 kb as estimated by representation of genes within large insert libraries. In addition, Steve Johnson presented work that suggests that the difference between male and female meioses is extensive in the zebrafish, with male meioses contributing 1/20th the number of cross-overs compared to the female. This should be taken into account when estimating the size of the zebrafish genetic map.

Fish Specific Technology

The zebrafish provides the means for systematically eliminating gene function throughout a vertebrate genome by producing a panel of deletion mutations that together uncover almost all chromosomal regions. Such a panel of deletions can be readily assembled by isolating and mapping gamma ray induced mutations. Individual deletions can be maintained as interbreeding heterozygous fish lines, as frozen sperm from heterozygous males, and as total genomic DNA samples prepared from haploid embryos. For example, a 96 well plate of DNA representing a genomic deletion panel would enable extremely rapid mapping of cloned genes or ESTs by PCR, without the need for identifying polymorphic strain differences.

To uncover the entire zebrafish genome (25 haploid chromosomes) roughly 100-200 deletions (of approximately 5-10 Megabases) are necessary. From the work of a few laboratories, deletions are already at hand for almost 30% of the genome; however, no systematic effort has been made to maintain these lines, to characterize in detail their homozygous mutant phenotypes, or to define the chromosomal locations and extent of the deficiencies. Moreover, to be effective and of general use to the community, such an enterprise requires a central repository to carry the deletion lines as fish and frozen sperm.

In the Halpern laboratory, the phenotypic analysis of deletions has been coupled with a PCR based approach termed “locus trapping”, which provides an efficient way to isolate specific genes and to scan the zebrafish genome in more detail for gene function. The procedure relies on collecting a large number of new deletion mutations induced in sperm at a high frequency (1/50), that break-up a preexisting deletion supplied by eggs from heterozygous mothers. Following in vitro fertilization, individual mutant progeny are identified by their phenotype, and although the new mutations can not be propagated if an essential embryonic gene is involved, total genomic DNA can be readily prepared from each mutant for PCR analysis. The DNA panel of mutant genomes is used to correlate the consistent absence of a specific genetic marker with a particular mutant phenotype. With this methodology, candidate genes can be tested for mutant phenotypes, new genes can be isolated with minimal need for time-consuming molecular strategies such as chromosomal walking, and the complex phenotypes of a large deletions can be broken down into their component single gene phenotypes. Although this approach relies on the unique properties of the zebrafish system (relatively short generation

time, production of large quantities of eggs, in vitro fertilization, and accessibility to mutagenesis and phenotype screening), the information gained from the systematic cataloging of loss of gene function will be of great benefit to functional studies of other vertebrate genomes.

Allele Screens and Insertional Mutagenesis

At the meeting, methods for the isolation of both new mutations and new alleles of existing mutations were discussed. For both candidate and position cloning approaches, it is advantageous to have multiple mutant alleles for the gene of interest. David Grunwald and Pacal Haffter described strategies for isolation of additional mutant alleles. Nancy Hopkins presented work on the isolation of new mutations by insertional mutagenesis (see Section IV below).

Research on the Zebrafish Genome

The goal of the meeting was to determine what resources need to be developed to support zebrafish genomics projects such as position and candidate cloning of mutant genes. A number of researchers stressed the importance of an EST (expressed sequence tag) project. Zhiyuan Gong presented the sequences of 1000 ESTs from the zebrafish. Database searches showed that 50% of the genes sequenced were related to known genes. Mark Fishman also discussed his lab's isolation of 900 ESTs. Both these efforts were on a small scale. The consensus in the genomics field is that for an EST project to be useful over 100,000 ESTs need to be sequenced. Marco Marra discussed how large scale EST sequencing projects in many different species are being done at Washington University. ESTs would be obtained for the zebrafish from multiple normalized libraries defining different stages of development as well as different tissues. A percentage of the ESTs could be placed on a map. Cheni Kwok described the recent construction of a zebrafish radiation hybrid panel. This panel can be used to map ESTs as well as candidate genes of interest to individual laboratories. It was agreed that in order for this panel to be useful to the community, a larger number of markers needs to be mapped onto this panel in the future. In addition, somatic cell hybrids (developed by Marc Ekker) could be used as genetic tools for positional cloning approaches. The resolution of the present zebrafish genomic maps was also discussed. Mark Fishman presented over 30,000 microsatellite cloned sequences which represent CA repeats. These CA repeats will be typed onto the framework map to generate at least a 2 cM resolution. In order to decrease the time needed to clone a mutant gene, it was suggested that the resolution of this map should be brought to 1 cM. It is possible to generate more markers throughout the zebrafish genome using mermaid-type repeats. This technique may be useful for investigators who are working on deletion allele mutations as well as for investigators who can obtain a somatic cell hybrid containing the single chromosome on which the mutation has occurred.

Summary of Work on Genomics

In a relatively short time, the community has amassed a number of critical reagents that will allow positional and candidate approaches to occur. Sufficiently polymorphic strains of zebrafish are available to allow genetic mapping and the current detail of the map will allow resolution of about 2.5 cM distances. The availability of large insert libraries and the generation of somatic cell and radiation hybrid panels should allow the fish to participate in genomics at a comparable level to human and mouse species.

There simply is not enough detail to the genetic map of the zebrafish. Additional markers are required for the requisite 1 cM distance that will allow positional cloning to occur. This could be done by increasing the density of microsatellite markers on the map. In addition, placing cDNAs on the map will take advantage of large regions of conserved synteny between humans and zebrafish.

Candidate cloning is an easy mechanism for isolating mutant genes. An EST sequencing project and mapping of cDNAs on radiation hybrid panels will provide a highly dense map that will be helpful for positional cloning approaches. Efforts on establishing fish-specific techniques for genomics would also be helpful.

Recommendations

100,000 ESTs should be sequenced and 10,000 to 20,000 of these genes should be placed on a genetic map using the radiation hybrid panels. For the EST project, there should be an effort to utilize normalize cDNAs libraries from multiple stages of development and from multiple tissues. Hans Lerach has generated some zebrafish libraries which have been normalized using an oligonucleotide hybridization technique. Other libraries may need to be created. In addition, a 1 cM map for microsatellites should be established. These could be obtained from the currently available 30,000 cloned microsatellites that Mark Fishman has generated. Regarding a framework physical map of the zebrafish genome, fingerprinting of the large DNA insert libraries (PAC or BAC) is possible for a small cost.

IV. An Insertional Screen

Currently insertional mutagenesis is probably the most rapid and cheapest approach to cloning embryonic lethal genes in fish. Applied on a large scale it could, within a few years, provide the field with a substantial fraction of the genes that are genetically essential to make a viable zebrafish embryo. It must be emphasized, however, that insertional mutagenesis is not a replacement for genomic approaches, but rather is a complementary approach. The lower

frequency of mutation relative to chemical mutagenesis, and the bias of insertional elements in their sites of integration mean that a positional cloning approach will also be essential in the zebrafish. However, the insertional mutagenesis could provide a large number of cloned genes in the near future, including genes for classes of mutants that were discarded in the chemical screens, and thus would provide insight into the many types of genes essential for the development of a vertebrate embryo. It was the sense of the meeting that this approach should be pursued vigorously.

Background

In invertebrate organisms, including for example Drosophilia, molecular cloning of mutant genes is facilitated by the use of insertional mutagenesis. The frequency of insertional mutants is usually considerably lower than that of chemical mutants, but mutants are readily clonable. Thus, a particularly powerful approach to forward genetics has been the combination of large scale chemical mutagenesis screens to observe all the mutant phenotypes that can be obtained, combined with an insertional mutagenesis screen to render some fraction of the genes readily clonable.

In the case of zebrafish, the power of insertional mutagenesis is considerable because of the large size of the genome, the current state of the fish genome project, and the fact that most chemically induced mutants are single base changes that were induced on non-inbred backgrounds. However, the number of insertions that are needed to hit all the genes in the zebrafish is large, theoretically on the order of several hundred thousand, and this makes the development of methods of insertional mutagenesis difficult.

This year one lab reported that mouse retroviral vectors with a VSV G-protein envelope could be used to infect the fish germ line and that the efficiency was sufficiently high that a small lab could easily generate several hundred thousand insertions. Mutants were obtained, and the mutated genes were readily cloned. It is estimated that a medium sized zebrafish lab applying this technology could isolate about 150 insertional embryonic lethal mutants per year.

Chemical mutagenesis screens reveal that there are very roughly 2400 genes required for the normal development of a zebrafish embryo. A mutation in any one of these results in death by about 5 days post fertilization. In the chemical screens to date, it is estimated that about half the 2400 genes were visualized. Of these, about 20% of all mutants displayed apoptosis in the CNS, about 50% displayed multiple developmental defects, and about 30% displayed quite specific developmental defects. Because of the difficulty of maintaining a very large number of mutants, and because of the virtual impossibility of cloning the chemically mutated genes at present, the vast majority of embryonic

lethals were discarded. In general, only those with specific defects were kept. It is probable the many genes involved in growth, cell cycle, and in diverse cell biological processes were discarded, while patterning genes were more likely to have been kept.

Since insertional mutagenesis renders mutated genes readily clonable, many of the mutants that are discarded in chemical screens could be analyzed if induced by insertional mutagenesis. Although the frequency of insertional mutants is lower than the frequency obtained with chemical mutagenesis, rapid gene cloning compensates for this difference. Furthermore, current insertional mutagenesis technology could probably be improved significantly. In addition, other methods of insertional mutagenesis might be devised. If successful, these improvements may ultimately make it possible for small labs to perform large scale insertional mutagenesis screens in the zebrafish.

Current technology: Insertional mutagenesis using mouse retroviral vectors.

Advantages
1. Ease of generating a saturation number of insertions in the germ line.
2. Rapid cloning of mutant genes. In the one lab where the technique has been developed and applied, 6 mutants were isolated in one year in a pilot screen and 4 of 6 mutated genes were cloned within that year.
3. Applied on a large scale, could lead to the cloning of a substantial fraction of the genes essential for embryonic zebrafish development within a few years.
Disadvantages
1. BL2+ facility required for containment of VSV-G pseudotyped viruses.
2. Low frequency of mutagenesis necessitates large scale screening. (About 1 in 70-100 insertions causes an embryonic lethal or visible mutation.)
3. Possible integration bias, since most insertional elements fail to mutate all possible genes.

Recommendations

1. Apply current technology

Fund laboratories to carry out large scale insertional mutagenesis screens using current technology.

2. Improve existing technology, develop new approaches

These goals would best be achieved via RO1s whose specific aims are to develop methods in the zebrafish, including insertional mutagenesis. New ideas and approaches still need to be tested. At present, grants proposing to develop technology can not compete in study sections where only hypothesis-driven research is well received. It is significant that the one method of insertional

mutagenesis so far developed for the fish received almost no support from the NIH. It was developed with support from private sources and NSF initially, and was only made possible by support from the industry.

(a) Improve existing technology using retroviral vectors as mutagens. Significant improvements to this technology could make it possible for even small labs to perform large scale insertional mutagenesis screens. These improvements could include: Higher titer virus stocks than used so far, resulting in more proviral insertions per F1 fish; vectors that are more mutagenic per insertion; gene trap vectors that use GFP as a reporter gene and thus allow one to identify fish with insertions into genes among the F1 progeny of founders, development of fish ES cells. In addition, the development of viruses that can infect fish cells but not human cells would eliminate or substantially reduce biohazard, and hence the need for a BL2+ facility, which limits the widespread use of the current technology.

(b) Develop new insertional mutagens and reagents. Decades of work have helped to elucidate mechanisms of integration and transposition of a number of different insertional elements. For some, the genes encoding the transposases or integrases and the target sequences required for insertion or transposition are now well defined. Some of these elements, for example, Tc1, can function in heterologous systems. Such elements might be injected into fertilized eggs and fish with insertions selected, or transgenic fish harboring elements that can be mobilized simply by mating fish might be developed. In addition, ES cells or equivalent technologies might be developed.

An exciting advance presented at the meeting by Shuo Lin was the tissue-specific expression of GFP in transgenic fish, including in F1 animals. These fish will have many uses, including in genetic screens using both insertional and chemical mutagens. This line of work should be pursued vigorously, both through RO1s and possibly an RFA mechanism.

V. International Collaborations

There is a large interest in Europe and Asia regarding the zebrafish as a model organism. This includes investigators in Germany, such as Christiane Nüsslein-Volhard, who is already maintaining a stock center at her own expense and who has made mutants freely available to the entire community with minimal input from the recipients. Obviously it will only be possible for this to continue with additional support for the stock center. Nüsslein-Volhard has also expressed interest in participating in a large scale insertional mutagenesis screen. In addition, Hans Lehrach has generated a genome effort for the zebrafish and is willing to provide help with libraries. Pascal Haffter is willing to place markers on the radiation hybrid panel as well as to increase the density of microsatellite markers on the current map. Zhiyuan Gong of Singapore has recently

sequenced 1000 zebrafish ESTs. He can obtain funding from the Singapore government which would be used for such an effort. A number of investigators from Canada are also interested in the zebrafish. This includes Marc Ekker, who had created somatic cell hybrids to position genes on the map.

Recommendations

For economical and scientific reasons, such international efforts in general should be encouraged.

VI. Role of Industry in Functional Genomics

The zebrafish community has not yet established general connections to industry, although individual labs are funded for particular projects. It is possible that companies utilizing a "functional genomics" approach would find the zebrafish an attractive system since many mutations could be easily created and the relevant genes cloned. In addition, it is possible to utilize the fish to affect disease processes. Some future examples of such work could include toxicologic or cancer screens in the fish. It is possible that industrial ties could be established to improve genomics and the ability to clone individual genes. One potential problem is how to protect the academic interests of the community once academic and industrial ties are established.

Recommendations

A task force of the zebrafish community industrial ties should be establish to identify guidelines on such interactions.

VII. Funding Support

The bulk of support for zebrafish research currently comes from the NIH. This includes RO1s and program project grants.

AGENCY	# GRANTS	TOTAL $
NIAMS	3	1,419,086
NCI	1	137,235
NIDCD	4	848,106
NIDR	1	36,302
NIDDK	11	1,180,230
NIEHS	7	731,173
NEI	8	917,168
NIGMS	4	518,561
NICHD	16	2,246,487
NHGRI	1	29,900
NHLBI	10	1,345,305
NINDS	10	1,561,128
NCRR	8	2,115,798
FIC	1	15,200
TOTAL NIH	85	$13,101,679
NSF	13 (24 PI'S)	$3,642,477

FEDERAL GRANTS FUNDING RESEARCH USING ZEBRAFISH.

National Center for Research Resources

The mission of the NCRR is to provide critical research technologies and shared resources that foster advances that improve human health. The NCRR recognized early on the potential of the zebrafish model for solving a wide variety of problems related to human health, and has made a critical investment in zebrafish research resources early in the development of the field, at a time when other funding was not available to provide the infrastructure necessary for the molecular genetic analysis of the interesting mutations that perturb zebrafish development.

The NCRR is funding the construction of zebrafish linkage maps, the comparative genomics of zebrafish and humans, the development of simple sequence repeat polymorphism markers, the production of embryonic stem cells which might lead to the key technology of knock-out mutations, and the cryopreservation of embryos, which would improve stock keeping. The NCRR is furthermore taking a leadership role in the quest for a National Zebrafish Stock Center, a facility that is crucial to the rapid exploitation of the genetic resources being developed in zebrafish.

In 1993 there were no two genes shown to be linked in zebrafish. NCRR funding resulted in the construction of a map with about 1200 anonymous DNA polymorphisms, and the fourth vertebrate whose genetic linkage map was closed (that is, whose linkage map contained the same number of linkage groups as chromosomes). NCRR support resulted in the remarkable discovery that, about the same number of chromosome translocations separate the zebrafish and human genome as separate the mouse and human genome. As a consequence of this fact, a researcher can move from a spot on the zebrafish genome to a corresponding region of the human genome. This then allows the zebrafish researcher to use the vast knowledge of the human genome to suggest candidates for zebrafish mutations. And reciprocally, when a zebrafish mutation is assigned to a gene that has a particular human orthologue, the zebrafish phenotype suggests a function for the human gene.

The tangible resources developed with the support of the NCRR will continue to be crucial for the efficient utilization of the remarkable promise of the zebrafish system to understand fundamental biology of vertebrates that underpins many human diseases.

Zebrafish Models of Disease

We asked individual investigators to prepare summaries on particular topics of zebrafish research. Each section includes highlights of the field, but is not meant to be all encompassing. Some areas of research have not been covered in this report, but additional information is available through the authors.

Recommendations

Each NIH Institute will embrace the zebrafish system in a different way. The number of grant applications on zebrafish will certainly rise dramatically over the next few years and funding through the RO1 mechanism will increase. A genome effort will improve the scientific success of each RO1, and further develop the field for the study of specialized gene function in vertebrates. For each field, it would be useful to have NIH Program Directors interface with the community and to familiarize the Institutes and Study Sections with the power of the zebrafish system. General reviews for each Institute could be written, and study section in-services could be done to aid this process.

Potential Contributions of Zebrafish to Cancer Research

Keith C. Cheng
Penn State University College of Medicine

The cancer cell frequently exhibits alterations in signal transduction pathways, cell-cell communication, and cell migration; these same processes are utilized during normal development.

A variety of mutants suggest relevance of zebrafish research to human cancer. Temperature-sensitive fin regeneration mutations were identified in a parthenogenetic screen done at the University of Oregon. The control of fin regeneration may involve basic cell growth control mechanisms that are relevant to cancer.

Among the mutants found in the Tübingen and Harvard screens are 22 complementation groups of mutants that affect hematopoiesis, all being studied at Harvard. Several of the mutants have a dysplastic syndrome reminiscent of myelodysplasia or pre-leukemia. Other mutants affect the induction of hematopoietic stem cells. Among the exciting research plans are the creation of zebrafish with leukemia and a screen for genes that suppress leukemia.

Xiphophorus is another fish system in which fundamentally important concepts of tumor suppression were established by the study of melanomas. Similarly, it is possible to look for tumor suppressing genes among zebrafish genes that are important in pigmentation. Towards such a goal, one can now use some of the nearly 300 pigment mutations in some 94 genes that affect neural crest-derived cells. The analysis of these mutations will undoubtedly shed light on the biology of melanoma, the most deadly of human skin cancers.

Programmed cell death, or apoptosis, plays an important role in both development and human cancer. A number of laboratories are investigating mechanisms of apoptosis and cell cycle control in both normal and mutant embryos. Whole embryo assays for apoptosis have been developed, and it is possible to screen for mutations that regulate apoptosis.

Loss of heterozygosity associated with the second hit in tumor suppressor genes is a fundamentally important genetic event in evolution of human cancers. In an ongoing screen at Penn State, supported by the National Science Foundation and recently by the National Cancer Institute, such mechanisms are being genetically dissected by the isolation of mutations that increase the frequency of loss of heterozygosity. These mutations cause spontaneous mosaicism in eye pigmentation in heterozygous mutant embryos. The mutants being identified will increase our understanding of the mechanisms by which loss of heterozygosity occurs in vertebrate somatic cells. These mutations are

expected to cause genomic instability, which leads to defects in chromosome segregation, recombination, and gene regulation.

Eye Development and Disease

John Dowling
Harvard Biological Labs

The zebrafish visual system, especially the eye, is particularly amenable for genetic analysis. Development of the eye is rapid; within 24 hours post-fertilization (pf) a well-formed eye is present. Differentiation of the neural retina occurs between 1 and 3 days pf; so that by three days pf the retina appears functional. Visual responses can first be elicited at this time, and by 5-6 days pf robust optokinetic reflex responses can be obtained from 98% of normal animals. Initially the retina is cone dominated; abundant rods are not evident until the second week of life. In adult fish, the retina contains four morphologically distinct cone types. Short single cones are ultraviolet-sensitive whereas long single cones are blue-sensitive. The principal and accessory members of the double cones are red- and green-sensitive respectively. In addition, the cones are arranged in a precise mosaic pattern across the retina, aiding in their identification.

In genetic studies carried out so far, numerous mutations affecting retinal development have been observed (Malicki et al., 1996; Heisenberg et al., 1996; Fadool et al., 1997). Most of these mutants were detected because of a small eye phenotype. Many show both retinal and brain defects histologically, but some show only retinal deficits. Mutants with abnormal retinal lamination have been observed, as well as mutants showing selective retinal cell loss. In some cases cells fail to form; in other cases, specific cell types rapidly degenerate after differentiation. Visual behavioral studies at 5-7 days (pf) (Brockerhoff et al., 1995; Brockerhoff et al., 1997) have revealed functional defects in morphologically normal fish. One mutant, for example, appears to have a defect in synaptic transmission between photoreceptor and second-order cells. Another mutant loses all its red-cones between 3 and 5 days pf, resulting in an animal deficient in red-sensitive vision. This latter mutation does not involve the opsin gene, suggesting that this mutation represents a new form of cone-specific color blindness. Finally, a dominant mutation causing slow photoreceptor cell degeneration has been detected behaviorally in adult fish (>4 months pf) (Lei and Dowling, in preparation). When homozygous, this mutation causes an early (~2 day pf) massive retinal and tectal cell degeneration and death of the animal by 5 days of age, suggesting that the gene involved is not photoreceptor cell-specific. Again, this mutation appears to represent a new type of inherited retinal degeneration. Another promising approach is the study of retinal-tectal projections. In Friedrich Bonhoeffer has been able to detect over 100 mutants which have defects of the ganglion cell axons finding their way to the tectum.

Future studies will include isolation of visual mutations using more subtle and sophisticated behavioral tests, as well as the further characterization

of both behavioral and morphological mutants. So far the focus has been on retinal mutations, but mutations affecting higher visual processing and eye movement mechanism are likely to be found. Molecular genetic studies to isolate the mutated genes represent an important next step in the enterprise.

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Organ Biology in Zebrafish

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One of the attractive features of the zebrafish is the ability to search for mutations that affect organ development.

Screens for organ mutants are of biologic and medical importance

1. In contrast to the physiology of the cell, there are no paradigms for the assembly or function of organ systems. What are the unitary steps for assembly of the heart and its integration into a seamless network of vessels? Are there mutations informative about the unitary elements of normal contractility and rhythm? How are organs rendered proportional to the mass of the whole animal?

2. Genes for fashioning organs are candidates for disease genes and for replenishment of failing tissues. The mutant genes may be those for disease propensity. Although recessive lethal in the screens, partial function in the human equivalent might render the organ susceptible to environmental insult. Screening for organ mutants is an approach to "functionalizing" the human genome project, providing key elements of important physiological pathways.

The zebrafish is the relevant genetic system for organ screens

Unlike *Drosophila* or *C. elegans*, the organs of zebrafish are similar to those of man. Whereas *Drosophila* lacks vessels and the heart has no ventricle, endocardium, valves, or conduction system, the zebrafish heart is essentially identical to that of the three week gestation human. The mouse embryo is not as good for organ screens because of its relative opacity. More importantly, and in contrast to the zebrafish, the mouse embryo depends upon ongoing delivery of nutrients and removal of waste products, leading to rapid deterioration of mutants which affect these processes.

Zebrafish organ mutants have proved important

1. Models of disease.

a) Congenital

The *gridlock* mutation lacks circulation to the tail, because of failure to assemble one branch point, embryologically identical to the region affected in congenital coarctation of the aorta. The asymmetry mutants, in which the right-left axis of the heart is reversed, affect the process believed perturbed in ventricular

inversion. The mutation with a diminutive ventricle affects processes relevant to the hypoplastic heart syndromes.

b) Adult

Many common disorders are impossible to model in animals, and are approachable in humans only by total genome scanning. For example, heart failure is a common and polygenic disorder, frequently "idiopathic" in nature. Several mutants evidence the same dilated poorly contractile physiology as do these patients. Other individual mutants model each common rhythm disorder, including heart block, fibrillation, and bradycardia. Mutants which cause disarray of epithelial patterning in the gut may be relevant to cancer, as are the mutants which affect angiogenesis. Those which perturb one pancreatic lineage speak to issues important in tissue replacement for cystic fibrosis and diabetes.

2. New paradigms in biology

These mutants have revealed steps of organ assembly, steps we could not have predicted to exist. In terms of generation of heart form, for example, there are genes needed specifically to generate one chamber but not the other, to assemble a single heart tube rather than two, to elicit valve formation or to generate the endocardium. Mutants separate lineages, such as those for pancreatic exocrine and endocrine cells. In each case, the mutation is discrete and other aspects of development are relatively intact. These mutants provide an entrance to pathways to understand development of the ventricle, or valves, or endocardium, etc. In addition, the mutants provide evidence that discrete genetic pathways regulate important physiological parameters, such as heart size.

Future programs

Biological and molecular work-up of current mutants

In vitro assay systems have been useful. Patch clamping of isolated cardiocytes, for example, has identified the channel defect in pacemaker mutants. Cell transplantation between mutant and wild-type fish has revealed the existence of unexpected signals, for example, how tissues regulate assembly of neighboring vessels. Positional cloning is underway. Using the microsatellite map, there is linkage for about 20 organ mutants, some now to BAC distance.

New screens

There are opportunities for two new types of screens with regard to organs. First, screens based on molecular probes can identify mutations in organ systems which are difficult to visualize directly, such as the kidney, or in the precursor populations which generate the organs. Second, insertional screens could provide a different category of mutant for study. Many mutants with pleotropic defects, including organs, were discarded in the first screen, because it was difficult to predict from phenotype alone how they could be informative. Many might have affected important genes, however, and would be quite useful and

interesting if the gene were known, as would occur more readily with an insertional agent.

Candidates for the human genome

It goes without saying that, once cloned, the genes for mutants with medically relevant phenotypes become candidates for disease genes. The phenotypes suggest that these might be especially useful in the study of complex and common diseases, for which uniform human populations may be elusive, such as heart failure, sudden death, and diabetes.

Hematopoiesis

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The zebrafish is an excellent system to study hematopoiesis. Blood is formed within 24 hours and the optical clarity of the embryo allows visualization of mutants that affect the number and differentiation of circulatory blood cells. Normal hematopoiesis occurs in a region above the yolk sac called the intermediate cell mass. This region yields primitive embryonic cells initially and then subsequently c-myb positive cells in the ventral wall of the dorsal aorta, similar to the aorta-gonad-mesonephros (AGM) region of higher vertebrates. The posterior region of the ICM contains cells that are poorly differentiated and have a blast-like morphology. These cells are likely to be stem cells or progenitors. Zebrafish globins have been characterized and embryonic to adult hemoglobin switching occurs after day 21 of development. Over 80 genes have been cloned that are homologs of human or mouse factors that participate in hematopoiesis. cDNA homologs have been isolated for every gene that has been knocked-out in murine embryonic stem cells which have a defect in hematopoiesis. This includes SCL, LMO2, GATA-1, GATA-2, TTG2, PU.1, myb, flk1, flt4, and fli1. Thus, the hematopoietic program has been largely conserved throughout vertebrate evolution.

The study of hematopoiesis in the zebrafish has been greatly helped by the availability of 24 complementation groups of mutants. These have mostly been isolated in the Tübingen and MGH screens, but also include newly derived mutants obtained at Children's Hospital, Harvard, and Oregon. Four of these groups have absolutely no hematopoietic stem cells and eight groups have defects in cell proliferation or differentiation. Five groups have a thalassemia-like disorder, although only one of these mutations is linked to the globin locus itself. This one mutant has an unstable hemoglobinopathy, and thus could be used to study the effects of pharmacologic agents to improve globin expression and stability. The other five groups are likely to have defects in globin regulatory genes. Transgenic zebrafish are being generated by Shuo Lin in collaboration with the Zon lab with the entire human globin YAC, and this can be used for screening the mutants for defects in hemoglobin switching. Two other mutants have porphyria. The two mutations that cause porphyrias could be used for drug screening or for studying the natural biology of this light-sensitive disease. Finally, we have isolated a mutant which is resembles a disease called CDA (congenital dyserythropoietic anemia), also known as HEMPAS. Thus, the mutations that could serve as models for human blood diseases include the thalassemia-like disorders, the porphyrias, and the group with CDA.

In the future, the genes for the currently available mutants will be cloned. Dominant suppressor screens can be done to isolate additional mutants that are

genetically interacting with these isolated genes. In addition, focused screens can be done for the absence of a particular molecular marker. For instance, a screen is being done for absence of globin expression at day 4. This should allow the isolation of all mutants that have a bloodless phenotype. Lymphoid screens are being done (by Willett and Steiner, at MIT and at Children's Hospital) for absence of lymphoid markers (such as rag1) and a myeloid screen could be envisioned based on absence of a myeloid-specific marker. It is possible to give the fish leukemia with dominant oncogenes and then search for suppressors of the phenotype. These suppressors would be excellent therapeutic targets for treating patients with leukemia. Finally, through use of transgenic zebrafish it is possible to study promoter or enhancer elements as shown by recent results of Shuo Lin. Using the GATA-1 promoter, he has created transgenic fish which target green fluorescent protein to blood cells. Isolation of the mutant genes will likely provide novel genes relevant to the to study of hematopoiesis in higher vertebrates and impact the study of congenital anemias, gene therapy, and bone marrow transplantation.

Genome Research

John Postlethwait
University of Oregon

The NHGRI is the major player in the Human Genome Project. This ambitious international undertaking seeks to develop materials that provide detailed information about the structure, organization and characteristics of human DNA in order to provide an understanding of the hereditary information that drives the development and function of a human being. Research on zebrafish can contribute towards achieving that goal. Zebrafish, of course, do not ride bicycles or build neutron bombs, but much of the development and physiology of zebrafish is fundamentally similar to human development and physiology, and most of the molecular genetic and morphogenetic processes that propel the acquisition of form and function in zebrafish are shared by humans.

Recent results show that zebrafish and humans even share large chromosome segments, which have been conserved intact during the 420 million years of evolution since the divergence of the zebrafish and human phylogenetic lineages. For example, the apparent orthologues of 12 genes localized along the full stretch of the long arm of human chromosome 2 (about 5% of the human genome) have been mapped in zebrafish. Nine of these genes reside on one zebrafish chromosome, and the remaining 3 on one other chromosome. This same group of 12 genes is also on two chromosomes in mouse. Analysis of the full set of mapped zebrafish genes suggests that the number of chromosome segments shared between zebrafish and human genomes may be about the same as the number of segments shared between the genomes of mouse and humans. The implication of this result is that comparative mapping should be almost as effective between zebrafish and humans as between mouse and humans. The pressing need now is to place enough orthologous markers on the zebrafish genetic map to clearly define the borders of conserved segments. This could be achieved by an active EST project and a good Radiation Hybrid mapping panel.

Rather inexpensive methods exist to develop physical maps. Restriction enzyme mediated "fingerprinting" of large-insert genomic clones and automated assembly of contigs has been a useful method to construct physical maps for C. elegans, yeast, and human chromosomes 7, X, and y. Given the genome resources already available for zebrafish, including the genetic map, and large insert Genomic clones, including YAC, BAC, and PAC clones. this technology could produce rather cheaply a map of sufficient extent to materially facilitate the isolation of genes disrupted by mutation.

The unique feature that zebrafish brings to the analysis of vertebrate genomes is the ability to perform an efficient mutational -- and hence a functional -- analysis of a vertebrate genome. If, for example, a zebrafish mutation maps to a chromosome segment known to be conserved between

zebrafish and humans, then the human genome can suggest candidate genes for the zebrafish mutation. Molecular genetic experiments can then test whether the candidate gene is in fact disrupted by the mutation. Reciprocally, and importantly, the phenotype of the zebrafish mutation can suggest functions for the human genes, which might otherwise be known only by sequence. In this way, a greater knowledge of the zebrafish genome could help move the human genome project from a descriptive to a functional stage.

Research on Aging

David Grunwald
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Zebrafish research will yield insights into genetic, cell biological, and developmental processes that influence or correlate with aging. Given the current scope of research activities in the zebrafish field, which emphasizes the study of the effects of mutations on development and cell physiology, we foresee four areas of ongoing research that may contribute significantly to our understanding of aging:

1) research concerning the accumulation of somatic mutations, including the study of genetic variants with altered somatic mutation rates
2) research concerning the biological effects of alterations in DNA metabolism, including the study of genetic variants that affect DNA replication, repair, or recombination
3) research concerning the biological effects of altered metabolic rates, including the study of genetic variants that display alterations in the accumulation of specific metabolite species, such as free radicals
4) research concerning factors that influence the rate of development, including the study of the potential correlation between the timing with which developmental landmarks are attained and other markers of aging or lifespan.

The areas of research highlighted here reflect the priorities of ongoing research in the zebrafish field. We anticipate that as the field matures and expands, a broader range of developmental and physiological characteristics will come under study, and some of these additional areas of research will be of significance to aging processes.

Research on Allergies and Infectious Diseases

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Similar to higher vertebrates, the teleost zebrafish has T and B lymphocytes and is thus an excellent system to study lymphopoiesis. Using degenerate oligonucleotide polymerase chain reaction (PCR) and low stringency hybridization, zebrafish homologs of early hematopoietic transcription factors, such as *c-myb*, PU.1 and *tal-1*/*scl*, as well as the immunoglobulin heavy chain gene (cμ), the lymphocyte specific marker *ikaros*, the T-cell tyrosine kinase *lck*, and *rag-1* and *rag-2* were obtained. Expression of the above genes in wild-type zebrafish assayed by in situ hybridization shows presence of T cells in the bilateral thymi as early as day 3 of life. The analysis of lymphocyte development is currently being extended to include 22 complementation groups of zebrafish with defects in blood production. These mutants potentially represent genes required for lymphopoiesis and are of particular interest as they are expected to have defects in stem cell differentiation at various stages. One of these mutants, *cloche*, appears to produce neither erythrocytes nor lymphocytes.

By analogy to erythropoiesis, where at least 22 complementation groups have been defined, multiple developmental steps are likely to exist between the uncommitted HSC and the mature lymphocyte. To derive a panel of mutants with defects in lymphopoiesis, we have carried out chemical and radiation mutagenesis of the zebrafish genome. We plan to screen these mutated zebrafish larvae with the erythroid and lymphoid markers, alpha-globin and *rag-1*, respectively. Mutants with defects in both markers are likely to have a defect in hematopoietic stem cell induction, proliferation, or differentiation. Mutants with defects in rag-1 alone are likely to solely affect lymphopoiesis. In a pilot screen of 24 clutches of ENU mutagenized offspring, one mutation was identified that led to a defect in globin expression and one mutant was identified with no rag-1 expression. In an expanded screen, it is expected that 10-20 different erythroid and lymphoid mutants will be able to be identified in the near future. Mutations will be mapped onto the zebrafish genome and the corresponding genes cloned. Investigation of zebrafish with defective lymphopoiesis will further our understanding of hematopoiesis and will have therapeutic impact for bone marrow transplantation, stem cell gene therapy, and leukemia.

Studies of Musculoskeletal and Skin Developmental Diseases

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Washington University School of Medicine
Sharon Amacher
University of Oregon

The zebrafish offers a number of advantages for studying the biology of the skin and skeletal/muscle systems. Because embryonic development is external to the mother and the embryos are transparent, development of skin, pigment cells, bones and muscles are easily seen in the living embryo.

One major area of research in zebrafish is development of the melanocyte pigment pattern in zebrafish. Abnormal melanocyte development or survival in humans leads to a variety of syndromes or diseases, including piebaldism, vitiligo, moles, and skin cancer. Studies of development of the adult zebrafish melanocyte stripes reveals that these cells are recruited from a population of undifferentiated precursors or stem cells that persist throughout the life of the fish. Mutations have been identified that affect how the precursor cells are established or later recruited to proliferate and differentiate. Other mutations affect migration of melanocytes, or the distance between stripes. More than one hundred mutations have been identified that affect the development of embryonic melanocytes.

The control of bone growth and regeneration has been investigated in zebrafish. Dozens of mutations affecting jaw cartilage or bone formation have been identified. Mutations affecting the regeneration of the adult fin may help us better understand the genetic control of bone remodeling and healing. For example, when adult fins are amputated, normally post-mitotic differentiated bone cells at the amputation plane are recruited to divide and form the regeneration blastema. Screens for mutations that affect fin regeneration have identified seven temperature-sensitive mutations that affect aspects of the regeneration or morphogenesis of the regenerating bone in the fin. Other mutants have been identified that affect the rate of growth of the fin ray, either causing abnormal morphology of the bone, or bones that grow too slow or too fast. Molecular characterization of these mutations may reveal previously unknown players in these processes shared among all vertebrates.

Important recent work in zebrafish has advanced our understanding of vertebrate muscle development and function. The isolation of a large collection of mutants affecting the development and function of muscles provides an important resource for investigation. These mutants affect a variety of processes including: somite formation (5 genes), horizontal myoseptum formation (11 genes), myoblast differentiation (3 genes), myofiber development and/or organization (8 genes), muscle tissue maintenance (4 genes), and locomotion (>40 genes). Although preliminary characterization has been done

on these mutants, further analysis and molecular characterization is important. For example, double mutant combinations must be made to understand gene interactions and to order the genes identified by mutation into a regulatory hierarchy. Furthermore, we must discover the molecular nature of the encoded gene products.

Significant advances have been made in understanding the organization of the segmental plate and the origin of somites in zebrafish. For example, slow and fast muscle precursors become specified very early in development (long before overt muscle differentiation) and then undergo dramatic morphogenetic movements to assume their final positions in the embryo. Indeed, some zebrafish mutations, particularly those that affect horizontal myoseptum formation, may identify components involved in specifying these two muscle cell types.

A wide variety of molecular markers that identify different regions of the developing somites and segmental plate have been characterized in zebrafish. One such gene is *her-1*, a homolog of the Drosophila pair-rule gene *hairy*. As in Drosophila, this gene marks the developing segments in zebrafish in a pair-rule fashion. In fact, *her-1* "prefigures" somite development by several hours, making it the earliest known marker of specified somites. Mutations have been isolated that disrupt the expression pattern of *her-1* in zebrafish embryos, and thus identify genes important for the initial specification of somites.

The molecular characterization of genes responsible for mutant phenotypes may identify genes involved in human disease. For example, zebrafish mutants that display muscle-specific degeneration may identify components involved in the destructive degeneration-regeneration process that occurs in human muscle dystrophies. Besides identifying genes involved in these diseases, these mutant lines would then become useful model systems to test potential therapies. Other mutants, such as the large number of genes affecting motility and locomotion, may help us identify genes involved in proper neuromuscular function and in human neuromuscular diseases.

Child Health and Human Development

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Because fundamental developmental genetic mechanisms have been conserved in all vertebrates, zebrafish provides a useful model for understanding many aspects of human development. At the genetic level, conservation is apparent from mutant phenotypes. For example, the zebrafish *no tail* gene is the homologue of the *Brachyury* gene in the mouse, and the zebrafish *valentino* gene is the homologue of the *kreisler* gene in the mouse. *Brachyury/no tail* mutants lack tail and notochord development in both zebrafish and mouse. *kreisler/valentino* mutants lack development of hindbrain segments r5 and r6 in both zebrafish and mouse. These are the first two cases in which two vertebrate species have been connected by functional (mutational) analyses of orthologous genes. In both of these cases the phenotypic analysis available in zebrafish has pointed to new understanding of the defects in the mouse. These initial two cases demonstrate that zebrafish is a useful system for understanding how genes encode early development in all vertebrates, including mammals.

Thousands of mutations are now available. During the next few years we are going to witness a large number of wonderful phenotypic analyses coming very rapidly with these new mutants. There will be studies of epistasis using double mutants, showing us how the genes interact along pathways of development, as has been so productive in recent years with both *C. elegans* and *Drosophila*. The most crucial need is to advance our knowledge at the genomics level. The genomics work will allow the phenotypic studies to be complemented with molecular genetic analysis. Mapping these new genes, and then identifying them molecularly is crucial, not only to understand how the genes are functioning in zebrafish, but also to allow us to further connect genetic patterning of development between zebrafish and mammals. We will almost certainly learn that many of the genes now known only by their mutant phenotypes in zebrafish have direct counterparts in humans. This will help us understand what is likely happening when these human genes function incorrectly or not at all, as in many types of congenital diseases.

Research on Ear Morphogenesis and Hearing Disorders

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Animal models are extremely valuable for learning the genetic basis of inner ear disorders. Because of obvious similarities in the pathology of humans and mice with inner ear defects, the murine model is particularly important model for genetic deafness. It is now evident from recent work with the zebrafish, that a second powerful animal model is now available for studying the genetic basis of inner ear development and function. In particular, the ability to trace the fate and lineage of early embryonic cells, the feasibility of large scale screens of mutants with early embryonic phenotypes, the ease of assaying the developmental effects of gene products produced ectopically from injected RNAs, and the possibility of constructing transgenic and chimeric animals all make the zebrafish a potentially valuable animal model.

Recent mutant screens have yielded over 75 mutations (in approximately 30 different genes)that primarily affect development of the inner ear. Mutations in at least nine genes affect morphology and patterning of the inner ear epithelium, including semicircular canal development and development of maculae and critae. Other mutants affect ear size, otolith formation, or initial specification of the otic placode. Many of the ear mutants also exhibit abnormal behaviors such as swimming in circles or upside down. Work on these mutants is only just beginning, and undoubtedly, further analysis will increase our understanding of inner ear development. One particularly well studied case is the *valentino* mutation, which is a homolog of the mouse *kreisler* gene. In both zebrafish and mouse, a malformation of the inner-ear is accompanied in mutant individuals by abnormalities in the hindbrain, the site at which the gene is active, suggesting conserved signaling mechanisms in Inner-ear formation.

The vestibular part of the ear of teleosts and mammals is remarkably conserved. Inner ears of both taxa have semicircular canals, a utriculus, a sacculus, and types of otolith structures; the main auditory responder in mammals (and birds), however, the cochlea, is absent in the teleost ear. Nevertheless, for auditory functions, teleosts appear to use the sensory epithelium of the sacculus and other structures that are primarily vestbular in mammals. Since the sensory epithelia of both vestibular and auditory structures of mammals and other vertebrates share a common basic tissue architecture of hair cells and supporting cells, mutants defective in such structures in fish will be relevant to studies of human sensory epithelial defects. Related to this previous point is the finding that some types of mammalian deafness are accompanied by impairment of vestibular function; such syndromes (e.g.,

Usher syndrome type 1B) are caused by defects in components of the sensory epithelium common to the vestibular and auditory regions of the ear. Moreover, different alleles of the same gene can cause loss of vestibular function without impairment of hearing, or loss of hearing without loss of vestibular function. Therefore, identification of mutants with purely vestibular defects (easily identifiable in zebrafish mutant screens) may ultimately be useful for identifying genes that have both vestibular and auditory functions in humans.

Even at first glance, many of the recently identified zebrafish phenotypes resemble human inherited auditory disorders. For example, branchio-oto-renal syndrome (*BOR*), mandibulofacial dystosis, craniofacial dystosis, Alpert syndrome and some others are associated with craniofacial abnormalities. Similar combination of phenotypes is obvious in the zebrafish mutations *quadro* and *little richard* which affect both otic vesicle and branchial arches. Likewise, selected genetic defects of both human and zebrafish auditory system are associated with pigmentation defects. In humans, wardenburg syndrome, piebaldness, vitiligo and universal dyschromatosis involve depigmentation. Among fish mutants *golas*, *piegus, mizerny*, *punktata* and others affect both auditory system and pigmentation. The current collection of genetically defective zebrafish strains, although already impressive, is likely to expand even more. New, creative screening methods offer opportunities to search for mutations affecting more and more specific developmental processes. In the area of ear research, particularly promising are screens for defects of hair cell development. Zebrafish lateral line hair cells can be visualized by immersing fish larvae in solution of a fluorescent dye for a few minutes allowing for efficient detection of many aberrations of this important cell type. As hair cells are essential for auditory perception of both fish and humans, this type of screening may have substantial medical importance.

In the coming years, the zebrafish system should be valuable in studying the aspects of the genetic basis of sensory epithelial and neuronal development relevant to human diseases affecting hearing and vestibular function. Additional mutant screens can be designed to search for behavioral abnormalities in swimming, in the startle response, and in response to auditory stimuli. Screens utilizing in situ hybridization will be able to identify a collection of mutants in which neural crest derivatives are deficient or in which particular gene expression is absent. Another exciting possibility is to devise mutant screens which detect abnormalities in the structure of the sensory epithelium or in the signal transduction response to auditory stimuli. These prospects should be a major factor in encouraging research on the basic development and sensory physiology of the zebrafish inner ear, and hence, the middle ear of all vertebrates.

Craniofacial and Dental Research

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Considerable progress has been made in identifying and analyzing mutations that affect craniofacial development in the embryonic zebrafish. This work will provide noteworthy opportunities for understanding the etiology of vertebrate craniofacial abnormalities. For example, 33 alleles of 19 genetic loci that affect the development of the zebrafish jaw and branchial arches were revealed by the Tübingen screen and an additional 48 alleles of mutations at 34 loci have been found by the Boston screen. Work in a number of other labs has revealed a variety of mutations that affect the patterning of the vertebrate head and the development of neural crest derived structures. There are also a number of pigmentation mutants with pleiotropic effects on other neural crest derivatives that will undoubtedly provide important insights into the mechanisms of lineage diversification within the neural crest, and the regulation of development of various crest derivatives. Finally, a number of mutants affecting motility that express pleiotropic effects on neural crest derivatives suggest that the functions of a number of other genes in crest development will be uncovered when analysis of these mutations proceeds.

The special attributes of the zebrafish embryo (e.g., its transparency and rapid development outside of the mother or an eggshell, the ability to label individual cells with lineage tracers and to follow them in living embryos by time-lapse, DIC- fluorescence- and confocal microscopy), combined with the growing library of mutants that affect craniofacial structures or the neural crest from which such structures are thought to arise, provide an outstanding resource for research on the mechanisms of normal development of specific craniofacial structures and other neural crest derivatives. Thus, the zebrafish embryo provides a tractable experimental system to analyze specification of developmentally distinct lineages derived from the neural crest and the development of jaw and branchial arch structures.

Although the zebrafish lack dentition in the mandibullar and maxillary bones, they do possess masticatory teeth. These teeth are embedded in cellular bone and project into the oral cavity at the level of the pharynx. They oppose the dorsal hyperkeratinized pad. The teeth are composed of acellular dentin and have anatomy similar to that seen in humans. The supporting bone is highly cellular, and shows evidence of rapid turnover. The maturation of these teeth occurs within the first week of development, and precedes ossification of the major chondroid bones. A screen has been initiated to identify mutants that affect the normal formation of these teeth. These mutations will represent interesting models for identifying precise molecular and cellular mechanisms of tooth development. The study of these mutants will be complimentary to work

done in higher vertebrates, and will likely provide novel genes that would not have been identified in those higher species.

Kidney Development and Diseases

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Zebrafish pronephric kidney mutants offer a unique opportunity to apply a genetic approach to the discovery of essential genes required for differentiation of epithelial cells and proper kidney function. Fifteen mutations have been isolated which give rise to cysts in the pronephros, resulting from defects in terminal epithelial differentiation.

The pronephros in zebrafish is a simple nephron composed of three parts: a glomerulus, a pair of pronephric tubules which drain the filtrate and the bilateral pronephric ducts which are analogous to the distal tubules and collecting system in higher vertebrates. Each of these pronephric structures are found to be affected in different mutants. For example, *double bubble* is one of 12 different loci that all develop cysts originating in the glomerulus and adjacent regions of the pronephric tubule. Ultrastructural analysis suggests that the glomerular basement membrane may be malformed in *double bubble* or missing a connective tissue protein. Interestingly, pronephric duct epithelial cells in *dbl* embryos fail to establish appropriate cell polarity as evidenced by an apical localization of the Na+/K+ ATPase, a normally basolateral membrane protein. The similar phenotypes of *double bubble* and 11 other pronephric mutants suggest that a genetic pathway regulating terminal epithelial cell differentiation may be revealed. The mutant *fleer* demonstrates a pleiotropic defect distinct from *double bubble*. Glomerular capillaries are dilated and the normally cuboidal pronephric tubule cells are squamous. A third mutant, *pao pao tang*, develops cysts in the pronephric tubules while the glomerulus and pronephric ducts do not appear to be affected. Currently bulked segregant mapping strategies and positional cloning are being used to isolate the affected genes in these mutants.

The range of affected tissues in these mutants, which in *fleer* also includes brain and liver, suggests parallels to autosomal dominant polycystic kidney disease in humans (1). Polycystic kidney disease (PKD) is one of the most common genetic diseases in humans, affecting roughly 1 in 1000 individuals (1). PKD can occur perinatally and result in severe kidney dysfunction leading to renal failure, or more commonly it can present as an adult disease with the progressive distention of several tubular segments or glomeruli, also leading to renal failure (1). Two genes, PKD1 and PKD2, which account for many of the cases of autosomal dominant PKD have recently been cloned(2-4). However, associated proteins and the signaling pathway downstream of these molecules remain unknown. The 15 zebrafish mutants being studied may shed light on the mulitlocus nature of cystic disease and help unravel the regulatory pathways involved proper terminal epithelial differentiation. Hereditary abnormalities in renal basement membranes resulting in glomerular dysfunction have been

detected in Alport's syndrome (5). Zebrafish mutants such as *double bubble,* which has basement membrane defects, may be relevant to this condition.

The mutants isolated so far were found by virtue of an obvious cyst; these mutations affect terminal cell differentiation and the later stages of kidney development. It is likely that, with the appropriate screening method, many more kidney mutants will be isolated with defects in all stages of kidney development. The group at Mass General Hospital is planning a new screen for mutations in organogenesis using in situ hybridization to detect more subtle and organ-specific phenotypes. We expect to be able to address the questions of early commitment to the kidney lineage, inductive signaling in kidney development, and angiogenesis in the glomerulus. Other more specialized screens could be undertaken. A prostate-specific gene has been isolated in the zebrafish, and it may be possible to screen for mutants that affect the genesis of the prostate.

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Developmental Toxicology In Zebrafish

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The zebrafish has been extensively utilized for toxicologic studies, allowing rapid testing of various agents on normal embryogenesis. The zebrafish system has the potential to genetically dissect pathways that regulate response to toxins.

The polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) are global environmental contaminants and potent developmental toxicants. Human exposure occurs during pregnancy and breast feeding when these chemicals are transferred transplacentally to the embryo and fetus and lactationally to the neonate posing a human health risk. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the prototype compound used to study this type of toxicity. In all vertebrates, the developing embryo/fetus is significantly more sensitive to TCDD than the adult, suggesting that TCDD has the ability to perturb critical developmental events. Therefore, a thorough mechanistic understanding of TCDD action during early development is necessary to assess the risk that such exposure poses to children. Toxic effects of TCDD occur by TCDD binding with high affinity to the aryl hydrocarbon receptor (AhR) followed by changes in gene expression. After TCDD binding, the activated AhR translocates to the nucleus and dimerizes with its partner ARNT. AhR-ARNT heterodimers recognize and bind dioxin response elements found in promoters of responsive genes to alter gene expression. AhR and ARNT are members of the basic helix-loop-helix/PAS family of transcription factors that include drosophila PER and SIM, murine ARNT-2, and human hypoxia inducible factor 1a. Members of this family are master developmental regulators, and it is intriguing to speculate similar roles for AhR and ARNT.

The rationale for studying AhR and ARNT function in zebrafish is that much of our current understanding of TCDD action involves characterizing endpoints of TCDD exposure, and very little is know about the actual mechanism of toxicity. It is largely accepted that TCDD developmental toxicity in fish like mammals is AhR-mediated, but the gene targets of activated AhR-ARNT that are causally related to the expression of toxicity are poorly understood. Furthermore, the understanding of the physiological role of AhR and ARNT in normal development is meager at best. The zebrafish are being used to investigate the developmental toxicity of TCDD and the physiological function of the AhR signaling pathway. Zebrafish embryos exposed to graded doses of TCDD for 1 hour, from 2-5 hours post fertilization, are responsive to TCDD (Toxicol. Appl. Pharmacol. 142: 56-68, 1997). The developmental toxicity is characterized by pericardial and yolk sac edema, craniofacial malformations, and mortality. Importantly, severe hemodynamic changes, manifested as slowed blood flow in vascular

beds of the trunk, head, and gills and slowed heart rate, occur prior to or coincident with the onset of lesions. Visual inspection of the transparent embryos shows that TCDD does not inhibit initial development of vasculature or prevent initiation of blood flow to the above structures. Rather it appears to interfere with the maintenance of peripheral vascular beds after they are formed.

Future research will be directed at determining whether the hemodynamic changes are due to a direct effect of TCDD on maintenance of the vasculature and/or are secondary to cardiac insufficiency. Mutagenesis screening will be used to identify key genes involved in the developmental cardiovascular toxicity of TCDD and for this purpose a zebrafish genetic map would be helpful. The human health significance of this research is that development and maintenance of a vascular supply is a fundamental requirement for organogenesis in the embryo. TCDD exposure does not appear to affect vasculogenesis. Rather the subsequent process of angiogenesis which includes sprouting, growth, migration, and remodeling of endothelial cells seems altered. Therefore, our future research will focus on the hypothesis that the developmental cardiovascular toxicity of TCDD is caused by an interference with angiogenesis. Angiogenesis is also implicated in the pathogenesis of a variety of human diseases such as proliferative retinopathies, age-related macular degeneration, tumors, rheumatoid arthritis, and psoriasis. Findings in zebrafish may ultimately demonstrate whether or not the AhR signaling pathway is an important regulator of angiogenesis with the degree of AhR activity in vascular endothelial cells correlating with physiological and/or pathological regulation of blood vessel growth.

Early Developmental Programs in the Zebrafish

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The zebrafish has emerged as an important model organism for the study of early development in vertebrates. Zebrafish and other vertebrates share many fundamental similarities in the organization of their body plans and developmental pathways. The experimental advantages of the zebrafish make it uniquely suited for the investigation of dynamic processes such as axis formation and gastrulation. For example, the optical clarity and external development of the embryo allow one to trace the movements of individual cells in the living embryo as the animal develops. Moreover, large-scale genetic screens have identified more than two thousand mutations that collectively define the functions of hundreds of genes essential for many aspects of early development. For example, *dino* and *swirl* disrupt neural induction and the dorsal-ventral axis, *bozozok* eliminates the Spemann organizer, *trilobite* alters the morphogenetic movements that reshape the embryo during gastrulation, and *spadetail, no tail, floating head* and numerous others perturb the development of specific cell types and organs that are established during embryogenesis. The elegant phenotypic analysis of these mutants that is possible in zebrafish will provide an unparalleled understanding of the functions of the disrupted genes. For example single-cell labeling experiments demonstrated that the *floating head* mutation alters the fates of a specific population of cells in the Spemann organizer, such that they form muscle instead of notochord. *floating head* is one of a handful of zebrafish mutations that have been cloned, and close homologs have been identified in other vertebrates, suggesting that these genes have the same function in pattern formation as defined in zebrafish.

In the vertebrate embryo, the notochord is known to produce ventralizing signals for patterning the overlying neural tube and the adjacent somites. A vertebrate homologue of the hedgehog gene from *Drosophila melanogaster* termed *sonic hedgehog* (*shh*) has received enormous attention as the most likely candidate for such a signaling molecule from the notochord.

In the zebrafish, four groups of mutants with defects in midline signaling have been described. The first group of mutants lacks a notochord and therefore fails to produce the ventralizing signal. The second group of mutants lacks a normal floor plate in spite of a morphologically normal notochord. A third group of mutants lacks the horizontal myoseptum and forms U-shaped instead of V-shaped somites. A number of experiments have shown that signaling from the notochord is required for the induction of the horizontal myoseptum and in fact, several of these mutants show both a somite and a floor plate phenotype. In

the fourth group of midline mutants, retinotectal axons project to the ipsilateral tectum instead of crossing the midline and projecting to the contralateral tectum as in wild-type. This retinotectal phenotype is also found in several of the mutants with defects in patterning of the somites and the neural tube.

Recently, one of the midline mutants was identified as a mutation in the *sonic hedgehog* gene of the zebrafish. Several experiments involving double mutant analysis and *in situ* hybridization experiments using target genes of Hedgehog signaling as probes imply that other mutants in this group affect genes encoding components of the Sonic Hedgehog signaling pathway. Elegant transplantation and injection experiments have also placed several of these mutants at different positions along the Hedgehog signaling cascade. These mutants will therefore be very useful for identifying new genes involved in Hedgehog signaling and for studying their function and interaction in the vertebrate embryo.

Recent studies indicate that several human hereditary diseases, including certain forms of cancer, are caused by mutations in genes involved in Hedgehog signaling. These include Grieg cephalopolysyndactyly syndrome (*Gli3* gene), Nevoid basal cell carcinoma syndrome (*PTC* gene) and familial holoprosencephaly (*shh* gene). Recent studies also suggest that cholesterol may be involved in modulating Hedgehog signaling, which might explain why inhibitors of cholesterol biosynthesis are potent teratogens. Hedgehog signaling was found to be involved in the differentiation of several organs and tissues such as cartilage differentiation or sperm development. The medical and economic importance of Hedgehog signaling has already been recognized and a number of companies are presently exploiting the therapeutic potential of Hedgehog proteins and other components of the Hedgehog signaling pathway. The zebrafish mutants described here will definitely provide a powerful tool for identifying new components of this pathway and for studying their function in vertebrates.

The molecular analysis of more of these mutations will identify many genes with essential functions in fundamental processes conserved in early vertebrate development. New screens will discover more mutants with developmental and behavioral phenotypes that have not been scored previously, and genetic modifier screens will define genes that interact with key players in established pathways. Further development of transgenic technology and refinement of imaging techniques will take functional analysis of these genes to a new level, and perhaps allow the in vivo detection of gene expression.

Mutants That Affect Mental Behavior

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The small size, optical transparency and rapid development of zebrafish make them good subjects for studying the mechanisms that regulate development of nervous system function and behavior. Several labs are currently funded to study retinal physiology including John Dowling, Harvard University, who is recording from cone photoreceptors and Douglas McMahon, University of Kentucky, who is investigating mechanisms of retinal synaptic plasticity by electrical recording. Joe Fetcho, SUNY Stony Brook, is recording membrane potentials in hindbrain neurons during escape behaviors using optical imaging techniques. John Schmidt, SUNY Albany, is studying activity and trophism in synaptic stabilization of retinal arbors. In principle, the results of these types of studies can provide not only important information about normal physiology, but also can be used to design genetic screens for mutations in genes that regulate these physiological processes.

The first screen for behavioral mutants was carried out by Kimmel and Westerfield, University of Oregon, in the 1980's and was based on a simple test of the response to touch. A number of mutations affecting neural crest cells (sensory neurons), motoneurons, and muscles were discovered. Additional alleles of some of these mutations as well as several new mutations were obtained in the recent large scale screens which looked for paralyzed or weak movement phenotypes.

During the past year, at least two labs have begun new genetic screens for mutations that affect behavior. The James Hurley lab, University of Washington, is screening for mutations affecting visual behavior and Kate Whitlock in the Westerfield lab, University of Oregon, is screening for olfactory mutants on responses to olfactants. The olfactory screen has already uncovered several new mutations. Further elaboration of behavioral mutant screens and molecular characterization of the disrupted genes will help further out understanding of the genetic basis of vertebrate behavior.

Neurogenesis and Neurological Disorders

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The study of neural development and function in the zebrafish is facilitated by the translucence and accessibility of the embryo, the relative simplicity of its nervous system, and the availability of a large number of mutations disrupting genes that are essential for normal development and physiology. Despite its simplicity, many of the features of the zebrafish nervous system are conserved in higher vertebrates and humans, including the patterning of the neural tube, the differentiation of neurons, the routes of axon tracts, neuronal signaling, and many aspects of behavior. Large-scale mutant screens in zebrafish have led to the identification of more than 50 genes affecting various aspects of neural development and function. The identified genes are involved in neural induction, anteroposterior and dorsoventral regionalization, axon pathfinding, neuronal differentiation, and behavior. Another 100 genes were found that are required for neural survival. To name a few examples,

-*cyclops* mutants lack ventral regions of the neural tube, resulting in cyclopia.
-*mind bomb* mutants display supernumerary neurons.
-*unplugged* mutants lead to the abnormal axon pathfinding of motorneurons.
-*who-cares* mutants affect the topographic mapping of retinal axons on the tectum target area.
-*nic* mutants are immotile and affect acetylcholine receptor function.
-*space cowboy* mutants display degeneration of the central nervous system.

The genes disrupted in these mutants are important entry points to understand the mechanisms of neural development and function e.g.; patterning of the neural tube (*cyclops*), control of neuronal cell number (*mind bomb*), axon pathfinding and connectivity (*unplugged, who-cares*), synaptic transmission and behavior (*nic*), and neural survival (*space cowboy*). In addition to their importance in the analysis of basic neurobiological processes, the identified genes might also have relevance to the study of disease states: defects in cyclopic mutants resemble the human congenital disorder holoprosencephaly, degenerative mutants might serve as models for neural degeneration conditions in humans, and retinal axon pathfinding mutants might serve as models for human defects such as ocular miswiring. The identified mutants define more than 100 essential genes by function, but only the future molecular isolation of the disrupted genes will allow deeper insight into the molecular mechanisms underlying neural function and development in this organism. Once cloned, powerful in vivo imaging techniques and further genetic screens can uncover the in vivo role of genes in zebrafish.

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