Making waves in cancer research: new models in the zebrafish

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The zebrafish (*Danio rerio*) has proven to be a powerful vertebrate model system for the genetic analysis of developmental pathways and is only beginning to be exploited as a model for human disease and clinical research. The attributes that have led to the emergence of the zebrafish as a preeminent embryological model, including its capacity for forward and reverse genetic analyses, provides a unique opportunity to uncover novel insights into the molecular genetics of cancer. Some of the advantages of the zebrafish animal model system include fecundity, with each female capable of laying 200–300 eggs per week, external fertilization that permits manipulation of embryos ex utero, and rapid development of optically clear embryos, which allows the direct observation of developing internal organs and tissues in vivo. The zebrafish is amenable to transgenic and both forward and reverse genetic strategies that can be used to identify or generate zebrafish models of different types of cancer and may also present significant advantages for the discovery of tumor suppressor genes that promote tumorigenesis when mutationally inactivated. Importantly, the transparency and accessibility of the zebrafish embryo allows the unprecedented direct analysis of pathologic processes in vivo, including neoplastic cell transformation and tumorigenic progression. Ultimately, high-throughput modifier screens based on zebrafish cancer models can lead to the identification of chemicals or genes involved in the suppression or prevention of the malignant phenotype. The identification of small molecules or gene products through such screens will serve as ideal entry points for novel drug development for the treatment of cancer. This review focuses on the current technology that takes advantage of the zebrafish model system to further our understanding of the genetic basis of cancer and its treatment.

INTRODUCTION

The zebrafish (*Danio rerio*) is a small tropical fish that has become a powerful animal model system for understanding the genetic basis of vertebrate development. The attributes of the zebrafish include its small size, fecundity, and production of optically clear embryos that undergo exceptionally rapid development ex utero. These qualities make the zebrafish amenable to forward genetic studies to dissect the molecular basis of developmental pathways as well as the phenotypic analysis of embryogenesis and organogenesis in vivo. In the decades since George Streisinger and his dedicated colleagues pioneered the genetically and experimentally tractable zebrafish system, it has proven its value as a model organism and contributed to our understanding of developmental processes (1–4). Furthermore, as the sequencing of the zebrafish genome reaches completion, it is also clear that there is a high degree of genetic conservation between man and other vertebrates despite millions of years of divergent evolution (5,6). This is reflected by the reiteration of developmental gene pathways and regulatory mechanisms. However, the zebrafish is only beginning to be exploited as a model for human disease and biomedical research. The same attributes that have led to the emergence of the zebrafish as a preeminent vertebrate embryological model are now contributing to its strong potential for clinical applications (7–11). Unbiased forward genetic analyses of zebrafish models of human disease provide the means to identify novel genes in pathologic pathways, as well as an unprecedented capacity for the in vivo analysis of disease processes and progression. This review focuses on the current technology that takes advantage of the zebrafish to further our understanding of the genetic basis of cancer and its treatment.

Cancer is a genetically complex disease that results from the multistep accumulation of somatic, and occasionally inherited, mutations that lead to clonal neoplastic cell transformation (12). The genetic lesions associated with cancer include the activation of dominant oncogenes and the inactivation of tumor suppressor genes through mutation and loss of heterozygosity (LOH). Mouse models have provided important insights into these collaborating genetic events that lead to cancer formation. For example, retroviral insertion screens and mating strategies using genetically manipulated mice have identified numerous genes involved in enhancing and suppressing the onset of leukemia and lymphoma (13–19). However, a limitation of these methodologies is that knockout strategies require the a priori knowledge of genes to inactivate,
and while retroviral insertion studies uncovered the overexpression of critical oncogenes, they have largely failed to identify inactivated tumor suppressor genes. Forward genetic screens using model organisms, such as Drosophila melanogaster and Caenorhabditis elegans, can overcome these limitations as indicated by their enormous contribution to our understanding of developmental and signal transduction pathways. However, invertebrate models are generally unable to recapitulate the pathogenesis of many human diseases. By contrast, the zebrafish system allows forward genetic approaches in a vertebrate that can manifest the pathologies of human diseases, bridging the gap between mammalian and invertebrate model systems. In addition, the establishment of transgenic lines expressing fluorochromes, such as green fluorescent protein (GFP), in specific developing tissues (20–25) makes the transparent developing zebrafish particularly amenable to in vivo studies of neoplastic progression, metastasis, and remission.

Cancer encompasses a wide range of heterogeneous tumor types that arise in different tissues, each with different molecular genetic signatures that often change as the disease progresses through different stages of malignancy. Furthermore, cancers exhibit lesions in many other genes that affect diverse cellular processes regulating cell cycle controlled growth and proliferation, genome stability and repair, telomerase activity, apoptosis, and tissue-specific differentiation. Exploiting unbiased forward genetic approaches will help to address the increasingly multifaceted genetic etiology of cancer. It is likely that the use of zebrafish cancer models in modifier screens will uncover a variety of novel genes and chemicals affecting diverse pathways that enhance or suppress features of the tumorigenic phenotype. These screens will lead to the identification of elusive tumor suppressor genes and pathways that are essential to pathogenesis in man. Once identified, such modifiers can serve as new entry points for the development of more efficient anticancer drugs and therapies.

There are three principal ways that cancer can be experimentally induced in zebrafish: (i) carcinogen treatment; (ii) transgenesis; and (iii) genetic predisposition induced by heritable mutations or insertions.

**ZEBRAFISH CANCER AND CARCINOGENESIS**

Historically, fish have been used as a model for toxicological and carcinogenesis assays (26–31). While it is difficult to document the extent of spontaneous fish tumorigenesis in the wild, upon exposure to aqueous carcinogen treatments they can develop a wide range of benign and malignant tumors. Interestingly, many of these fish cancers histologically resemble human tumors, suggesting the conservation of genetic mechanisms underlying the pathogenic changes associated with malignancy. In support of this, fish have many orthologs of the oncogenes and tumor suppressor genes that have been identified in mouse and humans,

![Figure 1. Generation of transgenic zebrafish.](image-url)

(A) Embryos are injected with transgenic cDNA constructs at the one-cell stage and grown to maturity (F0). These F0 animals are mosaic for the transgene, and when propagated, only those that underwent germline integration will yield offspring that express the transgene appropriately in the F1 generation. Due to mosaicism within the germine, only a percentage of the F1 offspring may express the transgene. While the expression of transgenes is dominant, positive F1 animals are heterozygotes and need to be incrossed to generate homozygous transgenic animals in the F2 generation. In this schematized example, a promoter of a ubiquitously expressed gene is used to drive the expression of green fluorescent protein (GFP; indicated by green color). (B–D) The progressive expansion of GFP-positive lymphoblasts in the zebrafish T cell acute lymphoblastic leukemia (T-ALL) model. T, thymus; E, eye.
including multiple *myc*, *ras*, and *notch* family members, β-catenin, *p53*, *mdm2*, *bcl-2*, and *bcl-xL* (32–34). Strong amino acid similarities and functional studies suggest the conservation of these proteins with their mammalian counterparts.

The range of carcinogen-induced zebrafish tumors can vary with the specific mutagen, indicating nonrandom activity. For example, the most prevalent type of tumors developing in fish treated with N-methyl-N'-nitro-M-nitrosoguanidine (MNNG) are hepatic neoplasms (35). MNNG can also induce mesenchymal neoplasms, including chondroma, leiomyosarcoma, and rhabdomyosarcoma, as well as epithelial neoplasms and tumors of the testes and blood, including seminoma, hemangiomia, and heman-giosarcoma. Other carcinogenic agents have also been used to induce tumor formation in the zebrafish, including 7,12-dimethylbenz(a)anthracene (DMBA) (7) and ethylnitrosourea (ENU) (36,37). DMBA exposure also results in a wide spectrum of tumor types, eliciting transformation of epithelial, mesenchymal, and neural tissues to form liver, gill, and blood vessel tumors (36). In contrast, ENU treatment acts more selectively, with most treated fish developing epidermal papillomas but no skin cancers (37). These fish also exhibit a low incidence of cavernous hemangiomia and malignant peripheral nerve sheath tumors. Taken together, these data demonstrate the ease of using zebrafish to assess carcinogenic responses in vivo and its capacity for developing a diverse range of cancers that pathologically resemble the heterogeneous tumor types present in man.

**TRANSGENESIS**

We have demonstrated transgenic modeling of cancer in the zebrafish by developing models of Myc-induced T cell acute lymphoblastic leukemia (T-ALL), proving that genetic predisposition to cancer can be stably acquired in this species (11). These transgenic zebrafish were generated by injecting cDNA constructs into embryos at the one cell stage, resulting in the tissue-specific expression of the cMyc transgene when integrated into the genome (Figure 1A). Because the enhanced green fluorescent protein (EGFP) gene was fused to the mouse cMyc transgene and targeted to the developing lymphocytes using the zebrafish *rag2* promoter, leukemia onset and infiltration could be monitored in real time (11). The EGFP-labeled leukemias developed in the thymus, spread locally into surrounding tissues, and eventually invaded skeletal musculature, visceral organs, and regions adjacent to the eye (Figure 1, B–D). The stable transgenic *rag2-EGFP-nMyc* zebrafish lines developed T cell leukemia with a mean latency of 22 days postfertilization (dpf) and a mean survival of 82 dpf. These observations highlight one of the key advantages of the zebrafish, allowing cancer progression and metastases to be analyzed in real time in an individual fish.

Zebrafish models can allow many critical assays to be performed that characterize a given cancer’s phenotype. For example, zebrafish leukemias were determined to be of T cell origin by demonstrating *TCR-α* gene rearrangement and expression of *lck* messenger RNA (mRNA) transcripts, while they failed to express immunoglobulin M (IgM) or to show rearrangements in the IgM locus. The zebrafish leukemias were shown to be oligoclonal by Southern analysis and DNA flow cytometry, demonstrating that some T-ALLs had increased DNA content. These data confirmed that the disease developed clonally from individual cells and required the acquisition of additional genetic lesions for full progression to T-ALL. The ability to transplant these leukemias into irradiated recipient fish showed that Myc-induced disease was due to clonal oncogenic transformation and not to an immune response. At the molecular level, Myc-induced leukemias are similar to a subtype of human T-ALL (38), expressing both *scl* and *lmo2*. In human patients, this subgroup represents the most common and most treatment-resistant form of this disease. Furthermore, as other zebrafish T-ALL model lines are developed based on other known contributing genes, the pathological effects of pathway interactions can be analyzed by selective mating strategies, as demonstrated in murine models (e.g., References 39–41). Thus, a zebrafish T-ALL model in modifier screens will likely lead to new insights in the molecular basis of this disease in humans.

Additional models of leukemia have been described using the zebrafish (42). The transient expression of the human RUNX1-CBF2T1, a fusion gene product of the t(8;21) translocation in acute myeloid leukemia (AML) in embryos, resulted in defective hematopoiesis, reduced circulation, and internal hemorrhages in the central nervous system and pericardium. These mosaic transgenic embryos also accumulated immature hematopoietic precursors and dysplastic erythroid cells in the posterior blood-forming region, highlighting the phenotypic similarities between mouse and zebrafish models of AML. Other transient transgenic zebrafish models of cancer developed in our laboratory include the targeted expression of human *MYCN* in the pancreatic islet, resulting in a number of animals presenting pancreatic neuroendocrine carcinomas between 4 and 6 months of age (43). Histological analysis of these tumors revealed lobulated arrangements of neoplastic cells expressing the *MYCN* transgene. The tumors expressed insulin mRNA and pancreatic exocrine cells and ducts were identified within the neoplasms, indicating the pancreatic origin for these tumors. Stable transgenic zebrafish lines expressing the RUNX1-CBF2T1 fusion protein and human *MYCN* will be valuable tools for the further study of genetic pathways contributing to this subtype of AML and to pancreatic neuroendocrine carcinoma, respectively.

In order to fully exploit the zebrafish as a human disease model, conditional transgenic strategies need to be firmly established. Disease-prone lines, such as the T cell leukemia model, need to be able to survive to sexual maturity and propagate well in order to be used further in modifier and enhancer/suppressor mutagenesis screens. To address this important technical issue, we and other zebrafish researchers are developing technologies using tetra-
cycline or estrogen responsive transgenes (e.g., MYC-ER™) or Gal4-UAS, CRE, and FLP recombinase strategies to drive the conditional expression of genes in the zebrafish. Establishing these methods will allow tumor-prone transgenic lines to be propagated disease-free and only exhibit the neoplastic phenotype when required. These strategies will also allow one to analyze the temporal effects of both transgene expression and inactivation on the tumor phenotype, which may give insights into their requisite roles at progressive stages of malignancy or during tumor remission.

GENETIC PREDISPOSITION TO CANCER IN ZEBRAFISH: FORWARD GENETICS

Over 70 years ago hereditary melanoma was first reported in a hybrid Xiphophorus (platyfish) strain that is genetically linked to the expression of a novel receptor tyrosine kinase (27,44,45). Thus, fish can exhibit a genetic predisposition to cancer that is accessible to forward genetic analysis. In the 1990s, two large-scale ENU-based mutagenesis screens in zebrafish were simultaneously performed at the Massachusetts General Hospital (Boston, MA) and the Max-Planck Institute (Tübingen, Germany), which resulted in the identification of thousands of mutants exhibiting a vast array of developmental phenotypes (46,47). These historic genetic screens set the precedent for all subsequent zebrafish screens, and we will discuss how three different types of mutagenesis strategies can be used to find mutations in genes relevant to cancer. These strategies are: (i) classic and gynogenetic haploid or diploid forward genetic screens; (ii) targeting induced local lesions in genomes (TILLING), a reverse genetic approach; and (iii) viral insertional mutagenesis screens.

The Boston and Tübingen screens were designed to identify recessive embryonic phenotypes in a classical F2 generation screen, which is similar in concept to the highly successful Drosophila screens conducted in the early 1980s to identify embryonic lethal
phenotypes (48). A subset of early developmental genes required for Drosophila embryogenesis were subsequently found to have human counterparts that function as tumor suppressor genes and oncogenes (49,50). Critical genes that function in the regulation of early development are often required later to control cell growth and maintenance in adult tissues (e.g., stem cells) and may contribute to the pathogenesis of cancer. In vertebrates, this is particularly evident, as a number of genes have been identified that function during organogenesis and are also disrupted in tumors of that tissue. These findings highlight the importance of developmental genes in cancer biology. Thus, the ability to identify genes in recessive embryonic screens in vertebrates should yield a number of novel genes that also function during tumorigenesis.

We are currently using a gynogenetic diploid screen to find mutations causing abnormal development of the embryonic peripheral sympathetic nervous system (PSNS) relevant to neuroblastoma. Neuroblastoma is the most common extracranial solid tumor of children and the leading cause of cancer death between the ages of 1–4 years (51). Studies have shown that microscopic neuroblastic nodules in 1 of every 250 infants (52–55) are formed in the PSNS between weeks 17 and 20 of gestation and regress around the time of birth. Haploinsufficiency or even total inactivation of one tumor suppressor gene may lead to this transient outgrowth of PSNS cells, but not be sufficient to sustain the partially transformed clone unless further mutations are acquired. It is believed that tumor regression may result from the normal activation of tumor suppressor pathways, induction of programmed cell death, or cellular differentiation. Furthermore, because neuroblastoma is the most common tumor already present at birth, the tumor suppressor genes contributing to neuroblastoma are among those most likely to also play critical roles during normal embryologic development. Thus, this cancer is an ideal target for zebrafish mutagenesis screens with proven strengths for identifying genes affecting embryological processes, such as PSNS development.

The specific genes involved in neuroblastoma are largely unknown. Cyto genetic LOH and comparative genomic hybridization (CGH) studies have identified chromosomal regions that are frequently deleted on one allele in neuroblastoma tumors (56,57). Chromosome band 1p36.1 deletions are found in about 35% of primary neuroblastoma samples, while allelic loss of nine additional chromosomal regions—2q, 3p, 4p, 5q, 9p, 11q23, 14q23-qter, 16p12-13, and 18q—have now been identified in 15%–44% of primary neuroblastomas (58). Unfortunately, the identity of nearly all target genes within these chromosomal regions remains unknown.

Our forward genetic screen was designed to assay for tyrosine hydroxylase (TH) expression in catecholaminergic neurons, allowing the identification of early defects in PSNS development. The screen is a modified version of the classic F2 screen and takes advantage of the ability to screen gynogenetic diploid zebrafish embryos, reducing the time and space required in the screening process by one generation (Figure 2). To perform this screen, male zebrafish are treated with ENU to induce point mutations in germ cells. The ENU-treated males are mated to wild-type females, giving rise to F1 progeny that are heterozygous for potential mutations that affect PSNS development. In general, ENU mutagenesis in zebrafish results in an average mutational inactivation of 100 genes/sperm (59). Assuming approximately 4 × 10^4 genes in the zebrafish genome, the analysis of 400 F1 mutagenized zebrafish should constitute one genome equivalent. The heterozygous F1 females are raised to maturity, and clutches of eggs are “squeezed” out of each individual F1 female, without killing the fish, and are then in vitro fertilized with UV-treated sperm collected from wild-type males. The UV-treatment cross-links and inactivates the sperm DNA so that it cannot contribute its genome to the developing zygote, however, the sperm cells remain viable and can activate the eggs. A French Press is used to apply “early pressure” to the activated eggs within 90 seconds following fertilization, which disrupts spindle formation during meiosis II and impairs the segregation of homologous chromosomes (60). This leads to gynogenetic diploid progeny, with up to 50% of the offspring being homozygous for alleles carrying ENU-induced mutations. The resulting embryos are then analyzed for abnormalities in TH expression by whole-mount in situ hybridization at 5 dpf. F1 females yielding clutches with abnormalities in TH expression are then outcrossed, and animals in the F2 generation are grown to maturity. The identification of heterozygous F2 fish carrying PSNS mutations is achieved by incrossing these fish and analyzing the resulting F3 clutches by in situ hybridization with the TH RNA probe. Assuming Mendelian inheritance and complete penetrance, a recessive mutant phenotype should be detectable in one-fourth of these F3 progeny if both parents are heterozygotes. This screen allowed the identification of mutants that either increased or decreased the numbers of neurons expressing TH in the developing sympathetic neurons (Figure 2, bottom panels). Once these genes are identified through established genetic mapping and positional cloning strategies (61), their human homologues can be determined and assessed for their relevance to neuroblastoma by evaluating whether these genes are mutated or exhibit LOH in human tumor samples.

**REVERSE GENETICS AND TILLING**

While the forward genetic capacity of zebrafish is well established, reverse genetic methods that alter the function of specific genes have recently become available that can aid in the development and analysis of cancer models. Genes of interest can be targeted by transgenic methods, as described above, or by transient gene knockdown approaches using antisense morpholino phosphorodiamidate oligomers (morpholinos). Morpholinos are designed to bind to the 5’ leader sequences or the first 25 bases 3’ of the translation start site. Unlike RNA interference (RNAi), morpholinos do not cause mRNA degradation through RNase H, but act by blocking translation and have proven to be more
specific and less toxic than many other antisense strategies (62). The injection of morpholinos directed against a gene of interest into zebrafish zygotes at the one- to two-cell stage produces an effective transient loss of function of endogenous genes that has yielded much information dissecting the molecular pathways of development. However, this method is generally restricted to studying the events in the first few days of development due to morpholino degradation. Successful stable gene knockout approaches through direct nuclear microinjection have been recently described in zebrafish, however these methods are technically difficult and have not yet garnered wide-spread use (63,64). A more robust reverse genetics strategy has been developed to perform target-selected mutagenesis, using the TILLING method. This method was first established in plants (65), but has since been widely applied using various chemical mutagens in model organisms, from C. elegans to mouse (66,67), and is now being used by our laboratory and many other zebrafish groups.

Briefly, a library of genomic DNA extracted from randomly mutagenized individual animals is screened for mutations in a specific gene of interest (Figure 3A). In zebrafish, ENU is used to mutagenize male animals, which are outcrossed to give rise to heterozygous F1 mutants. Typically only the F1 males are used in this screen, since their sperm can be cryopreserved for future reconstitution of the mutant line once relevant mutations are identified. This allows the genomic DNA library to be screened repeatedly in the future and still be able to recover corresponding mutant lines. The screen for mutations can be performed by either direct sequencing of the gene of interest or through the use of the CEL I nuclease, an enzyme that recognizes and cuts DNA heteroduplexes (68), to identify single base pair differences between the wild-type and mutant alleles of the target gene. When mutations are identified in the target gene, the mutant zebrafish line is recovered through in vitro fertilization of wild-type eggs using the corresponding cryopreserved sperm samples. The TILLING approach has already proven its utility in zebrafish, and a variety of mutant lines have been isolated affecting genes involved in immunity (rag-I) (69), microRNA processing (dicer I) (70), and the Wnt pathway’s role in heart development (apc) (71).

Relevant to zebrafish cancer models, our laboratory participated in a collaborative effort that successfully used TILLING to identify two zebrafish tp53 mutant lines (72). TP53 is the most frequently mutated tumor suppressor gene in human cancer, with nearly

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**Figure 3. Reverse genetic targeting induced local lesions in genomes (TILLING) method.** (A) Adult zebrafish males are treated with ethyl nitrosourea (ENU) and mated to wild-type females. The resulting F1 male progeny, containing approximately 100 mutations in each germ cell, are grown to breeding age and sacrificed to create a “mutagenesis library” consisting of: (i) genomic DNA, used to screen for F1 males harboring mutations in genes of interest and (ii) cryopreserved sperm from each individual male fish, for recovering the mutant lines using in vitro fertilization procedures that result in 50% heterozygous mutant animals in the F2 generation. The F2 carriers are incrossed to generate an F3 generation in which 25% of the offspring are homozygous for the mutation. This approach identified two zebrafish tp53 mutant lines. (B and C) Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assays demonstrating the induction of apoptosis by γ-irradiation in embryos at 30 hours post-fertilization (hpf). (B) Extensive apoptosis in the head and trunk of wild-type embryos is observed when exposed to γ-irradiation (16 Gray) at 24 hpf. (C) Apoptosis is suppressed in tp53 mutant embryos following this irradiation treatment. The adult tp53 mutant zebrafish developed a malignant peripheral nerve sheath tumor (MPNST), shown in the eye (D) and by histopathologic section (E).
50% of all tumors exhibiting a loss-of-function mutation. As a key regulator of the cell cycle and apoptosis, TP53 acts as a critical tumor suppressor by activating cell death in response to a variety of cellular insults that can result in the accumulation of detrimental somatic mutations leading to tumorigenesis. More than 90% of identified TP53 mutations in human cancers are found in the highly conserved DNA-binding domain, encoded within exons 4 to 8 in both man and zebrafish (73–75). We screened a library of 2679 fish by direct sequencing of these exons (69) and isolated five point mutations, two of which were similar to those found in human cancers, which functionally inactivate the tp53 protein. As in mouse Tp53 models, homozygous mutant embryos from these two lines failed to up-regulate p21 following γ-radiation treatment and subsequently did not arrest at the G1/S cell-cycle checkpoint. Unlike wild-type animals, mutant tp53 embryos also failed to undergo irradiation-induced apoptosis, another hallmark of TP53 tumorigenesis (Figure 3, B and C).

Interestingly, one of the mutant lines is temperature-sensitive, and cells only lacked cell-cycle arrest and apoptosis at elevated temperatures (37°C versus the normal 28°C) (72). As in mouse, homozygous mutant fish are viable, but beginning at 8.5 months of age they began to develop malignant peripheral nerve sheath tumors (MPNSTs) (Figure 3, D and E). MPNST is a rare neoplasm in wild-type zebrafish populations, but it has recently been found in a number of heterozygous zebrafish lines carrying mutations in different ribosomal protein (RP) genes (see below and Reference 76). The shared tumor phenotype of our tp53 line and the RP lines is currently being investigated to examine the possible interactions between these pathways and to provide new insights into MPNST. These zebrafish tp53 cancer models now provide a unique platform to perform modifier screens, which can identify genetic mutations or small molecules that act through tp53-independent pathways to restore apoptosis, cell cycle delay, and tumor suppression following DNA-damaging events.

**INSERTIONAL MUTAGENESIS SCREENS**

Despite advances in zebrafish genomics, mutant gene identification through positional cloning can still be time-consuming. In contrast to ENU, which usually induces single-base changes in DNA, insertional mutagenesis with retroviral vectors introduces exogenous DNA tags within sites of integration (77,78). Retroviral insertion in zebrafish usually leads to the loss of gene function rather than overexpression due to insertions into promoters that are often identified in mouse retroviral screens. Thus, zebrafish retroviral screens are an attractive alternative to large-scale ENU-based screens and substantially increase the speed with which candidate genes can be cloned (79). Using inverse PCR, one can readily clone genetic DNA flanking the retroviral insert and sequence it. These sequences are then used to search databases for regions of homology that can allow the rapid identification of the disrupted gene.

Recently, an impressive large-scale insertional mutagenesis screen in zebrafish was completed that identified 525 embryonic lethal mutations, representing approximately 390 different genes (80). Extrapolating from the number of genes that have been cloned, the authors hypothesize that approximately 25% of the embryonic lethal genes in the fish genome were identified in their screen. These mutants were initially identified by their abnormal embryological morphologies, and to further analyze affected genetic pathways, the authors are currently reexamining their collection through “shelf screens” using cell lineage-specific assays. The power of the shelf screen lies in the fact that the mutated gene is already known, eliminating the need for subsequent positional cloning strategies. A shelf screen examining genes required for normal kidney development has already been completed (81), and screens examining myeloid and PSNS development are also underway.

In the course of establishing and maintaining these insertional mutant lines, several were identified that developed malignant spindle cell tumors resembling MPNSTs seen in other species of fish and in mammals (76). Further analysis revealed that nearly all of these lines were heterozygous for mutations in different ribosomal protein genes required for protein translation. The retroviral insertion reduced or eliminated the expression of individual ribosomal proteins in the heterozygous animals, suggesting that these proteins function as novel tumor suppressors. This observation also demonstrates the ability to use heterozygous mutant zebrafish in dominant screens to identify tumor suppressors that act by haploinsufficiency or after tumor cell-specific LOH for the normal allele. Although these fish are predisposed to develop MPNSTs, they also develop other tumor types, including retinoblastoma and lymphoma. Ribosomal proteins are not a well-known class of tumor suppressor genes in humans, but with the high degree of conservation in regulatory pathways among vertebrates, mutations that inactivate ribosomal proteins may prove to be a new class of mutations leading to human tumors, further demonstrating the power of forward genetic screens in cancer research.

**CANCER MODIFIER SCREENS**

The ultimate goal of developing zebrafish cancer models is to be able to exploit them to conduct modifier screens, combining the ability to model human cancer pathologies with the capacity for powerful forward genetic analysis in this organism. Forward genetic modifier screens are well-established in invertebrate model systems and have generally been used to identify genes that regulate pathways responsible for a previously identified trait or phenotype (82,83). In modifier screens starting with zebrafish disease models, mutations in putative modifier genes would be identified that alter the disease phenotype, and in the case of cancer, would affect tumorigenesis, including selective neoplastic parameters such as latency of onset, tissue specificity, rates of disease progression, or metastases.

Disease models usually represent animals that already exhibit a “sensi-
tized" phenotype, such that enhancer and suppressor effects can be identified more easily. Such sensitized phenotypes would include zebrafish lines exhibiting a strong genetic predisposition to cancer, such as the T-ALL and MPNST zebrafish models (5,40,72). Modifier screens based on these animals could uncover the cooperative somatic mutations whose acquisition contributes to the multistep clonal evolution leading to tumor formation. The advantages of forward genetic screening based on a disease phenotype are particularly attractive because they allow a complex disease like cancer to be addressed genetically, despite incomplete knowledge of the many diverse cellular pathways that can contribute to the overt disease. The identification of these mutations will help unravel the many genetic pathways whose interactions contribute to cancer. Such modifier screens may take the form of a classic ENU-based mutagenesis screen using the tumor model line as the starting F0 fish (see Figure 2) so that recessive mutations affecting the phenotypic parameters of the tumor model line can be detected. Alternative modifier screens may be designed to identify dominant-acting mutations by crossing F1 animals, heterozygous for mutations, or retroviral insertions (see Figure 2, and the Insertional Mutagenesis section), with tumor model lines and assaying for alterations in the tumor phenotype.

Genetic mutations that enhance a cancer phenotype can identify either traditional recessive or haploinsufficient tumor suppressors (84). The loss of a haploinsufficient tumor suppressor would directly act as an enhancer of a cancer phenotype due to inactivation of a single allele and would be evident in heterozygous carriers of the mutation. However, the loss of one allele of a recessive tumor suppressor would only lead to an enhancer phenotype upon loss of the second allele either in homozygous mutant animals, if they are viable, or by tumor-specific somatic mutation or deletion of the normal allele (LOH). These types of mutations would contribute to the loss of cellular controls over proliferation, apoptosis, and differentiation. In contrast, mutations resulting in the suppression of a cancer phenotype are likely to result from the loss of one or both alleles of genes that are necessary for oncogenic transformation. Relatively few suppressors of malignancy have been identified in murine models, whether acting cell autonomously or non-cell autonomously, making this a promising area for research in the zebrafish system.

To be effective, disease modifier screens must overcome several obstacles. First, mutant or transgenic lines that are predisposed to develop cancer must be viable through sexual or non-cell autonomous or non-cell autonomy in murine models, whether acting cell autonomously or non-cell autonomously, making this a promising area for research in the zebrafish system.

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Lastly, the need to use statistical analyses to define a phenotypic feature of tumorigenesis, such as the time of tumor onset, can make genetic mapping and cloning of the mutated gene much more difficult. Thus, once a mutant line affecting tumorigenesis is identified and recovered, it may be important to find an alternative early phenotypic assay for that mutant line that will be more experimentally amenable to genetic cloning.

![Figure 4. An example of a chemical modifier screen. Zebrafish developing green fluorescent protein (GFP)-labeled T cell acute lymphoblastic leukemia (T-ALL) are arrayed into multiwell plates at the beginning of disease manifestation as defined by infiltration of GFP-positive lymphoblasts into regions adjacent to the thymus. Compounds from a small molecule library are added to the wells, and changes in fluorescent pattern and intensity are observed as the leukemia spreads throughout the animal. A compound that suppresses leukemogenesis reduces GFP expression by causing reversion to the wild-type phenotype. (Adapted from Reference 88.)](image-url)
techniques (e.g., embryonic lethality, tissue-specific effects on apoptosis, differentiation, or proliferation). Fortunately, inactivating mutations in most dominant modifier genes affecting neoplastic transformation are likely to result in recessive lethal embryonic phenotypes due to their participation in essential developmental functions. However, the detection of a distinctive embryonic phenotype as an alternative to the cancer phenotype, observed in the original dominant modifier screen, is not guaranteed and is therefore a limitation of this approach.

CHEMICAL MODIFIER SCREENS IN ZEBRAFISH

In addition to its power in forward genetic screens, the zebrafish is now emerging as a new model for drug discovery (Reference 85 and Figure 4). Chemical screens have been completed identifying small molecules that perturb normal development in the zebrafish, with some compounds having limited, or tissue-specific, effects (84). These elegant screens pave the way toward in vivo-based drug screens designed to bypass specific developmental defects. Peterson and colleagues have recently identified a compound which suppresses the gridlock mutation (86). gridlock mutants carry a mutation in the hey2 gene resulting in the disruption of aortic blood flow and early embryonic lethality. Remarkably, the chemicals identified in this small molecule screen caused up-regulation of vascular endothelial growth factor (VEGF) and are likely to function downstream of hey2 by affecting angioblast specification and migration. Treatment of developing gridlock mutant embryos with these small molecule inhibitors resulted in restoration of normal heart development and allowed embryos to survive to adulthood.

These experiments highlight several advantages of using the zebrafish for drug discovery. The small size of zebrafish and their capacity for generating large numbers of model animals make them exceptionally amenable to high-throughput chemical screening technologies. Zebrafish chemical screens can be conducted in vivo, allowing the identification of chemicals that require metabolism for conversion into active drug forms. Conventional in vitro cell-based chemical screens would fail to identify drugs that require metabolic activation within an organism. As in genetic screens, in vivo zebrafish assays allow, but are not restricted to, visual assays to monitor cancer phenotypes, such as GFP-labeled tumor formation and progression. In addition, conventional drug screens often focus on identifying specific inhibitors of an established molecular target. For example, imatinib (Gleevec®) was designed to target the ABL kinase, blocking signal transduction pathways downstream of the BCR-ABL fusion kinase in chronic myeloid leukemias (87). By contrast, reversion of a disease phenotype in the zebrafish does not require the a priori knowledge of gene targets. This allows the identification of drugs that disrupt novel pathways or affect the interactions of complex molecular signaling cascades that can contribute to disease states. Furthermore, it is likely that multiple drugs may be identified that act on different proteins within a common signaling cascade. Thus, drug resistance could be circumvented by treatment with multiple drugs that target different proteins in a given pathway.

The zebrafish holds immense promise for developing drugs to treat cancer. For example, drug screens designed to restore apoptosis and tumor suppression in tp53-deficient embryos will provide new lead compounds that could potentially inhibit a wide range of cancers dependent upon loss of functional tp53. In conjunction with radiation and chemotherapeutics, these drugs would be designed to restore apoptotic responses that normally are required for tumor cell death after treatment with cytotoxic drugs and radiation. Similarly, in conjunction with transgenic approaches, it may be possible to identify chemicals that curb disease by inhibiting oncoprotein function directly or by blocking downstream effectors of activated oncoproteins. For example, leukemic rag2-EGFP-mMyc transgenic zebrafish can be screened for small molecule inhibitors and assessed for leukemia regression, as determined by loss of GFP-labeled blasts or an arrest in disease progression (Figure 4).

CONCLUSIONS

The strengths of the zebrafish cancer model rely upon its capacity for use in forward genetic and chemical modifier screens and the ability to directly visualize tumorigenic processes in vivo through the use of fluorochrome-mediated technologies. As the technical challenges to fully exploit this model are addressed, researchers will be able to capitalize on the many strengths of this model for gene discovery in cancer research. ENU-based mutational screens and retroviral insertion strategies can provide a new understanding of the mutations and, ultimately, the pathways that suppress or enhance oncogenic transformation. Similarly, zebrafish cancer models possess enormous potential for high-throughput chemical screens to identify suppressors of tumorigenesis. These combined approaches promise to identify new genes and pathways that have important implications for improved understanding of the molecular pathogenesis of cancer and that provide critical entry points for the development of improved therapeautic drugs to combat this devastating disease.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES


