

Zebrafish: A Renewed Model System For Functional Genomics

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*In the post genome era, a major goal in molecular biology is to determine the function of the many thousands of genes present in the vertebrate genome. The zebrafish (*Danio rerio*) provides an almost ideal genetic model to identify the biological roles of these novel genes, in part because their embryos are transparent and develop rapidly. The zebrafish has many advantages over mouse for genome-wide mutagenesis studies, allowing for easier, cheaper and faster functional characterization of novel genes in the vertebrate genome. Many molecular research tools such as chemical mutagenesis, transgenesis, gene trapping, gene knockdown, TILLING, gene targeting, RNAi and chemical genetic screen are now available in zebrafish. Combining all the forward, reverse, and chemical genetic tools, it is expected that zebrafish will make invaluable contribution to vertebrate functional genomics in functional annotation of the genes, modeling human diseases and drug discoveries.*

Key words zebrafish, functional genomics, mutagenesis, transgenesis, gene trapping, gene knockdown, gene targeting, morpholino, chemical genetics

1 Zebrafish genome, mutagenesis and transgenesis

The zebrafish (*Danio rerio*) was developed as a vertebrate model system because of its advantages in transparent and external embryonic development.^[1,2] The zebrafish AB strain is the best characterized and most widely used strain in research laboratories (Fig. 1A). Most recently, an adult-transparent zebrafish strain named "Sheer" zebrafish has been developed (Fig. 1B), allowing for direct visualization of internal organs or fluorescently marked cells in adult zebrafish (unpublished data, personal communication with Dr. Henry Tomasiewicz).

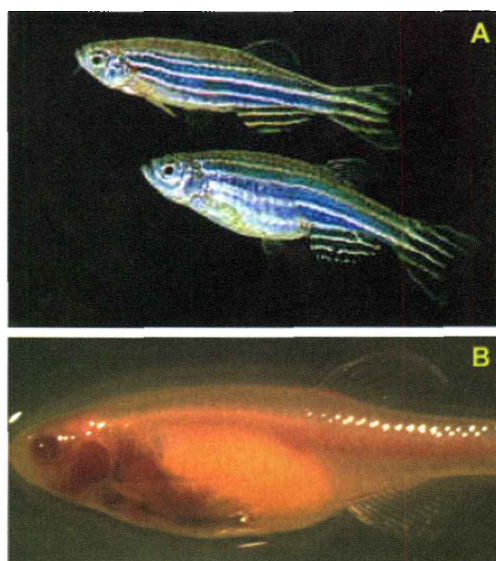


Fig. 1 Adult male (top) and female (below) zebrafish (A). Female transparent "sheer" zebrafish with egg visible within the belly (B). The picture of "sheer" zebrafish was kindly provided by Dr. Henry Tomasiewicz.

The zebrafish genome consists of $\sim 1.7 \times 10^9$ bp of DNA, which is organized into 25 pairs of chromosomes. It is estimated that the zebrafish genome contains $\sim 30\,000$ genes, which is 20% more than that of the other known mammalian genomes.^[3] To promote zebrafish as a model organism, the Wellcome Trust Sanger Institute started the Zebrafish Genome Project in 2001 to sequence the entire zebrafish genome (http://www.sanger.ac.uk/Projects/D_rerio/). The zebrafish Zv8 genome assembly was released in June, 2008 and it is expected that the Zebrafish Genome Project to be completed within the next two to three years.

Taking advantage of the large numbers of progenies from breeding, large-scale ENU (N-ethyl-N-nitrosourea) mutagenesis in zebrafish was performed in a few labs, resulting in combined of over six thousand mutant strains.^[4,5] In these studies, the ENU mutagenesis was performed in male zebrafish (spermatogonia) and the phenotypes were screened in F3 progenies for mutations.^[4,5] These early studies screened for developmental phenotypes including abnormalities of gastrulation, epiboly and axial patterning in the early developmental stages as well as abnormal development of notochord, brain, fin, retina, heart and blood. Many mutations resembling human disease states have also been identified. More than 500 genes might be defined by complementation analysis in these mutations.^[6] However, it is laborious to identify mutant genes by positional cloning in ENU-induced mutants (Table 1). To facilitate the cloning of the mutant genes by inverse PCR, a more sophisticated pseudotyped retrovirus based insertional mutagenesis method has been developed, leading to the identification of 315 developmentally regulated genes in zebrafish in one screen.^[7,8]

The first few zebrafish transgenic lines were generated by microinjection of plasmid DNA into the newly fertilized eggs about two decades ago.^[9,10] However, the early technology in generating transgenic zebrafish by microinjection of naked DNA is hampered by low frequency of transgene germline transmission and unreliable expression of the inserted genes.^[11] The transgenesis frequencies are increased by an approach to flank the transgene with I-SceI restriction sites and co-inject the vector with the I-SceI meganuclease.^[12] The use of fluorescent transgenic reporter such as green or red fluorescent protein (GFP or RFP) allows for visualiza-

tion of gene expression through the embryonic developmental stages without sacrificing the animal.^[13,14] These fluorescent reporters are now widely used in zebrafish, including GFP or RFP fusion genes.^[15]

The discovery of two vertebrate transposon systems, *Sleeping Beauty* (SB) and *Tol2*, has enabled the generation of transgenic zebrafish at a very high efficiency.^[16,17] The SB and *Tol2* transposon systems consist of a transposon DNA vector and a transposase mRNA, which are delivered by microinjection into one to two-cell stage zebrafish embryos. The SB and *Tol2* vectors integrate into the genome by a “cut and paste” mechanism at a high efficiency when delivered together with their transposases.^[18] It is generally believed that SB and *Tol2* transposons integrate into the genome in a random fashion. Though the SB transposon is reported to always integrate at the TA nucleotides, the *Tol2* element has no specificity for its integration sites at the DNA sequence level.^[19] SB and *Tol2* transposons have now been used widely in generating transgenic zebrafish.^[20-25] Most recently, a third transposon system, the *Ds/Ac* elements, has also been successfully used in zebrafish with high frequencies of transgenesis.^[26] Unlike the SB and *Tol2* transposon systems, the *Ds/Ac* elements are of plant origin. The maize *Dissociation* (*Ds*) element contains two terminal repeats but no transposase gene and it depends on *Ac* element or *Ac* transposase for transactivation. The *Ac* element is an autonomous transposon carrying a transposase gene between the cis-acting terminal sequences that contain 11-bp imperfect terminal repeats. The efficient transposition of maize *Ds/Ac* in zebrafish genome suggests that *Ac/Ds* elements do not rely on host specific factors for transposition.^[26]

2 Transposon-mediated gene-trap mutagenesis in zebrafish

Gene-trap mutagenesis is a technique to identify gene function and expression patterns by random insertion of reporters into the genome. This strategy is successfully applied in mouse mutagenesis to “trap” developmentally regulated genes,^[27] enhancers^[28] and promoters.^[29] An International Gene Trap Consortium (IGTC) has been formed to introduce insertional loss-of-function mutations across

Table 1 Zebrafish Functional Genomics Tools and Their Applications

Levels of Manipulation	Technology	Feature	References
DNA	ENU mutagenesis	Random mutation induced by chemical; all phenotypes; positional cloning to identify the mutated genes	4,5
	Transgenesis	DNA microinjection into fertilized egg; fluorescent marker; transposon or I-SceI meganuclease to facilitate transgenesis	9,12,13,16,17,26
	Trapping based mutagenesis	Includes enhancer, promoter or gene trap; random mutation; genome-wide; easy to identify mutated gene; often delivered in transposon systems	21—25
	TILLING	Combines ENU mutagenesis with targeted PCR sequencing; identify point mutations of selected genes	48,49
	Gene tagging	Functional knockout of selected genes; reverse genetics; using zinc finger nuclease (ZFN) technology	54,55
mRNA	Site-specific recombination	Cre-loxP recombination system	15,61,62,69
	Inducible gene expression	Spatial and temporal control of gene expression by heat chemical, prodrug, laser, light or transcriptional factors	73—75,77,78
	Morpholino	Antisense-based gene knockdown technology using synthetic oligonucleotides, delivered by microinjection	35,36,41—43
Protein	RNAi	Down-regulate gene expression by microRNA	44,45
	Chemical genetic screen	Developing embryos for screening; often fluorescently labelled; small molecule drugs; in vivo screen	82—88

the mouse genome (<http://www.genetrap.org/>). However, the mouse gene trapping is inefficient as compared to similar strategies in zebrafish because mouse gene trapping is conducted in embryonic stem (ES) cells,^[30] involving complicated embryo transplantation processes. Because zebrafish embryos develop externally within water, by employing fluorescent reporters such as GFP or RFP, the gene trapping events can be directly visualized under the microscope. The transparent and rapid zebrafish embryonic development (3 days) allows for quick identification of gene-“trapped” zebrafish in early embryonic developmental and organogenic stages (Fig. 2).

While the gene trapping strategy “traps” the exons upstream (5'-exon trap) or downstream (3'-exon trap or polyA trap) of the transgene insertion site, the enhancer trapping is designed specifically to identify tissue specific regulatory enhancer elements.^[30] The polyA trapping was not used as often as promoter and 5'-gene trapping in mice because of the existence of a mRNA-surveillance mechanism, nonsense-mediated mRNA decay (NMD), which resulted in biased vector integration into the 3' most intron of the gene. This problem in mouse polyA trapping has been solved by vector modification with the insertion of an IRES sequence before the SD (splicing donor) site.^[31] The NMD mechanism is conserved in eukaryotes.^[32-34]

Transposon-based gene and enhancer trapping methods using *SB* or *Tol2* transposon have been developed and tested in zebrafish,^[20-25] which is illustrated in figure 2. A high-efficiency mutagenic gene trapping vector, the Gene-Breaking Transposon (GBT) system containing a 5'-mutagenicity cassette and a 3'-gene finding cassette, was developed for zebrafish mutagenic gene trapping screen.^[25] The GBT-based vector was successfully used to generate a recessive loss-of-function mutation at the *Tnni2* locus, resulting homozygous zebrafish without a heart beat (silent heart mutation, personal communication with Dr. Stephen Ekker). Our lab has recently performed a zebrafish gene trapping pilot screen and identified trapped lines with tissue-specific EGFP expression in developing zebrafish brain, muscle fibers and heart (Fig. 2).

3 Zebrafish gene knockdown and TILLING

The zebrafish embryos develop very fast; it only takes three days for a fertilized zebrafish egg to develop into a newly hatched fry. This developmental feature makes antisense-based morpholino knockdown technology very efficient in developing zebrafish embryos.^[35,36] Morpholinos (MOs) are chemically modified synthetic oligonucleotides that can hybridize to the single-stranded nucleic acid sequences with high affinity.^[37] MOs are stable and not subject to any known endogenous enzymatic degradation.^[38] To down-regulate gene expression, MOs are usually designed to sterically block the pre-mRNA splicing or protein translation. Since the first report of the efficient MO gene knockdown of ubiquitously expressed GFP transgene, phenocopying of gene mutations in zebrafish and the development of MO-based zebrafish models of human diseases,^[35] many studies have been published to down-regulate gene expression in zebrafish early embryonic patterning, cardiovascular, neural and pancreatic development, and many other developmental processes.^[35,36,39-43]

Another gene knockdown technology that has been successfully used in zebrafish is RNA interference (RNAi), which involves microRNA molecules.^[44,45] MicroRNAs are non-coding single-stranded RNA molecules of 21–23 nucleotides in length that are processed from small hairpin RNAs (shRNA). Their function is to specifically silence the gene expression by degrading the targeted mRNA molecules.^[46,47]

However, as the gene knockdown is manipulated at mRNA level (table 1), this down-regulation cannot hereditarily pass onto the next generation. To generate mutations into specific genes in the genome, a reverse genetics tool termed Target Induced Local Lesions In Genomes (TILLING) was developed.^[48] This target-selected mutagenesis strategy combines ENU mutagenesis in generation of F1 mutant zebrafish library with nested PCR and direct sequencing in identification of the mutated genes. TILLING has been successfully used to generate a library of 4608 ENU-mutagenized F1 animals and screened for mutations of 16 genes. 255 mutations were identified in this study.^[49]

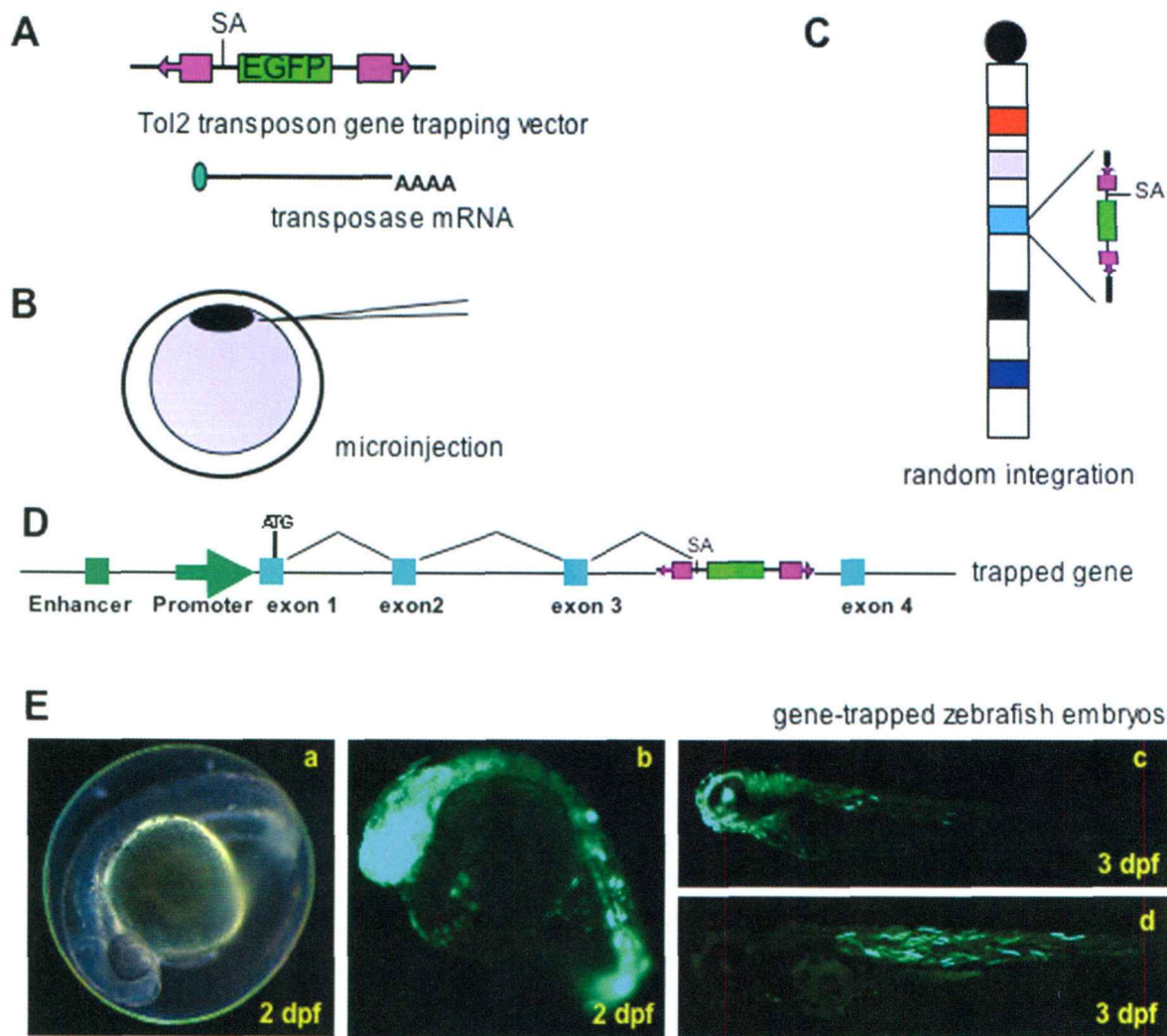


Fig. 2 Transposon-mediated genome-wide gene trapping. The gene trap vector carrying a splicing acceptor site (SA) and the transposase are delivered into the newly fertilized zebrafish egg by microinjection (A&B). The vector randomly integrates into the genome (C) and occasionally into gene (D). The upstream exon of the insertion will splice onto the transgene and the EGFP will be expressed. 2–3 days after microinjection the expression pattern of the injected zebrafish can be visualized under fluorescent microscope (E).

4 Zebrafish gene targeting

The mouse gene targeting strategy utilizes a mechanism of homologous recombination in embryonic stem (ES) cells.^[50-51] ES-like cells have been isolated in zebrafish,^[52] and the cultured zebrafish ES cells have the ability to contribute to germ-lines.^[53] However, this strategy has not yet been used to successfully generate targeted gene knockout in zebrafish. The zebrafish gene knockout technique has been recently developed using a completely new targeting strategy, the Zinc Finger Nuclease (ZFN)-based knockout strategy.^[54,55]

ZFNs are designed proteins consisting of engineered zinc finger DNA-binding domains linked to the catalytic domain of the FokI restriction endonuclease. Based on the zinc finger array chosen, this engineered enzyme can cut the target gene and generate mutation through DNA repair of the double strand break (Fig. 3). In zebrafish gene targeting, the ZFNs are delivered as mRNA by microinjection into fertilized zebrafish egg. The two simultaneously published reports both demonstrated that the delivery of ZFNs resulted in targeted gene knockout and germline transmission at a high frequency (>25%).^[54,55] While Doyon *et al.* se-

lectively targeted the zebrafish *golden* and *no tail/Brachyury (ntl)* genes, Meng et al. knocked out the zebrafish ortholog of the vascular endothelial growth factor-2 receptor, *kdr*. Both groups observed small deletions and insertions at the knockout loci.^[54,55] The limiting factor in deploying this technol-

ogy in zebrafish is the availability of the custom-designed ZFNs although the standard protocols for how to generate them were published.^[56-59] Nevertheless, it is anticipated that ZFN-based gene knockout technology will generate many invaluable zebrafish models for human diseases.

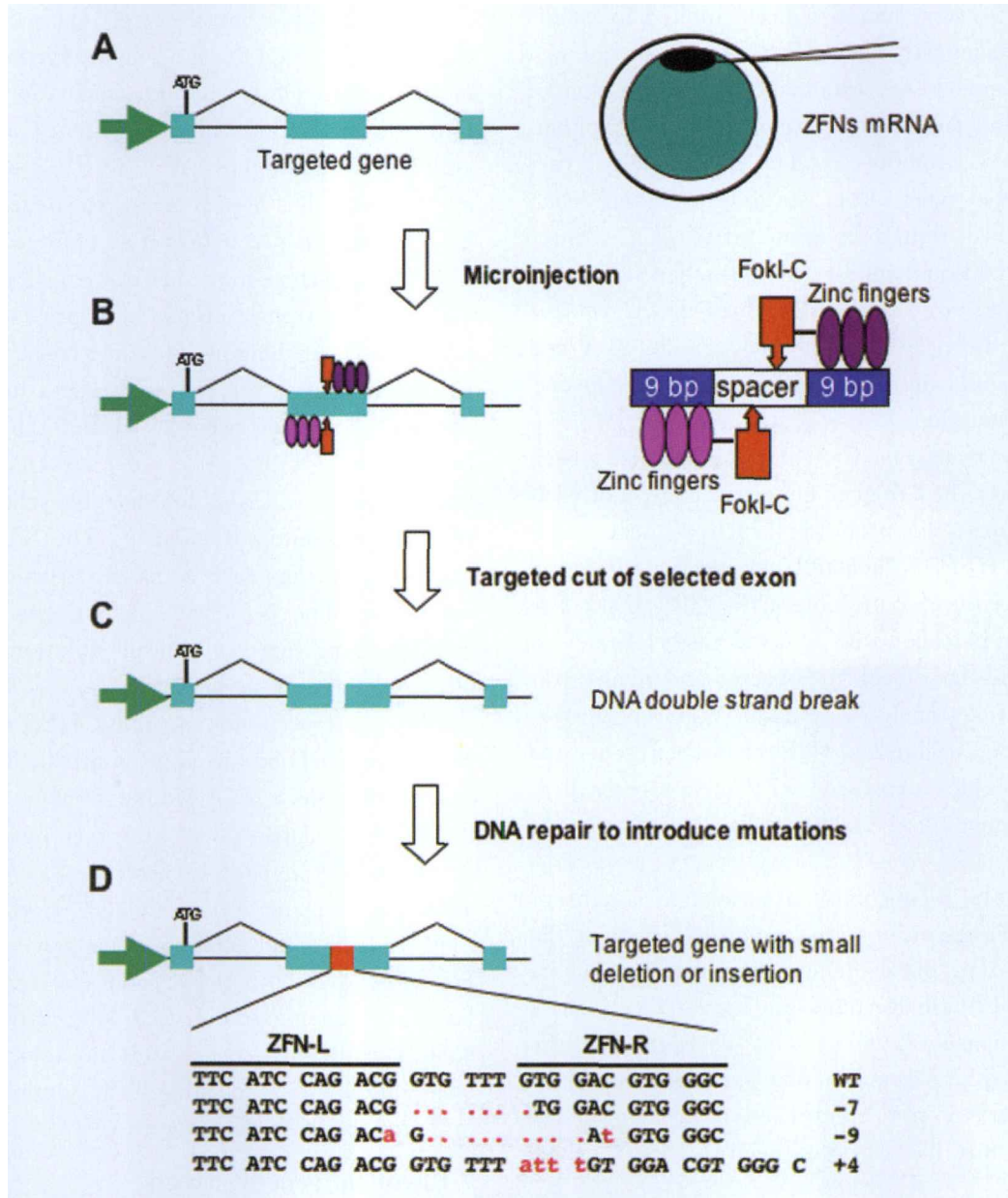


Fig 3 Zebrafish gene targeting by Zinc Finger Nucleases (ZFNs) technology. The mRNAs of the ZFN arrays were microinjected into one-cell zebrafish embryo (A). The ZFN proteins were translated, bound to the targeted region and cut the DNA (B&C). Cell DNA repair introduced mutations with small deletions or insertions (D).^[55]

5 Site-specific recombination systems for conditional mutagenesis in zebrafish

Cre is a bacteriophage site-specific recombinase which catalyzes the recombination between

two *loxP* sites (34 bp consensus sequences). The *Cre/loxP* system has been widely used in generating conditional transgenic mice or conditional gene knockout mice.^[60] Cre recombinase has also been shown to be an extremely potent enzyme in zebrafish.^[61-64] Pan *et al.* generated a floxed (*loxP*

flanked) GFP transgenic line with the floxed GFP controlled by the muscle-specific *mylz2* promoter. After microinjection of the *in vitro* synthesized Cre mRNA into the floxed *mylz2:GFP* embryos, expression of GFP was dramatically reduced and the deletion of GFP was confirmed by PCR.^[61] The Cre/*loxP* system has also been applied in conditional mutagenesis in zebrafish. A zebrafish T cell leukemia model was established by expression of *mMyc* transgene under the control of a lymphoid-specific *rag2* promoter.^[65] The transgenic fish carrying *mMyc* were often severely diseased when they reached reproductive maturity age. Therefore, a conditional approach was further pursued to generate a *rag2-loxP-dsRed-loxP-EGFP/mMyc* transgenic line. In this line, *mMyc*-induced T cell leukemia could only be generated after Cre-mediated DNA excision.^[62]

A few transgenic Cre zebrafish lines have been created with the Cre gene under the control of various tissue-specific promoters such as heat shock promoter *HSP70*, hematoblast-specific *Mlo2* and oocyte-specific *zp3* promoters.^[66-68] To make Cre expression pattern to be detected easily, Cre/GFP fusion genes have been constructed and demonstrated to be functional in zebrafish.^[15] A reporter zebrafish line, designated G2R, has been generated recently, which expressed RFP upon Cre-mediated excision of loxP-flanked GFP gene (green to red).^[69]

The Flp-*frt* recombination system is another site-specific recombination system. It involves the recombination of sequences between two short (22 bp) Flipase Recognition Target (*frt*) sites by the Flipase enzyme (Flp) derived from the yeast.^[70] This recombination system is functionally conserved and highly active in mammalian cells^[71,72] but has not yet been reported in zebrafish.

6 Inducible gene expression systems

Transgenic lines with genes of interest be regulated in both spatial and temporal manners would be especially useful. Zebrafish heat shock promoter *hsp70* has been cloned and used to control GFP expression in transgenic zebrafish. At normal temperatures (28.5°C), GFP was not detectable in transgenic embryos, but was robustly expressed

throughout the embryo following an increase in ambient temperature (37°C).^[73] Furthermore, laser-induced gene expression in specific muscle fibers was successfully performed under control of the *hsp70* promoter.^[73]

RNA caging is another technology to spatially and temporally control gene expression. The caged-mRNA by 6-bromo-7-hydroxycoumarin-4-yl methyl (Bhc) almost had no translational activity but recovered transcriptional activity after uncaging by illumination of UV light.^[74] The photocaging technique has been reported to silence zebrafish gene through RNAi in zebrafish embryo.^[75]

The galactose-inducible system of yeast, mediated by the transcriptional activator Gal4 and its consensus UAS binding site, has been demonstrated to efficiently trap zebrafish genes in a self-reporting trap vector design.^[76] Using a bacterial nitroreductase (NTR) gene under the UAS control, tissue-specific cell ablation has been achieved with the Gal4 regulatory system.^[76] The NTR enzyme converts prodrugs such as metronidazole (Met) to cytotoxin. The NTR-Met system has also been employed to achieve cell lineage ablation in pancreatic α cells and cardiomyocytes.^[77,78] Most recently, a mifepristone-inducible LexPR system has been developed and tested in zebrafish. This vector system contains a DNA-binding domain of the bacterial LexA repressor, a truncated ligand-binding domain of the human progesterone receptor and an activation domain of the human NF- κ B/p65 protein.^[79] Using this system, Emelyanov and Parinov generated both driver and effector zebrafish lines and demonstrated that the transgene was strictly controlled and could be induced at any stage of the life cycle through the administration of mifepristone in the fish water.^[79]

7 Chemical genetic screen

Chemical genetics is a relatively new scientific discipline that has emerged over the past 10 years and refers to the use of small molecules to affect biological functions.^[80,81] Similar to the classical forward and reverse genetics, chemical genetics can result in many phenotypes or biological traits which may lead to elucidation of biological functions or drug discoveries. Zebrafish is particularly suitable for chemical genetic screening because of

its large number of progenies from breeding, the small embryo size, and the rapid and external embryonic development. Zebrafish embryos are permeable to small molecules, allowing for easy drug administration in the fish water. Comparing to traditional cell-based screens, zebrafish provides a whole vertebrate system for drug screening that combines the biological complexity of *in vivo* models with the ability for high throughput screening.^[82,83] The use of developing zebrafish embryos for drug screen also provides a quick assessment for potential toxicity *in vivo*.

Several chemical genetic screens have been carried out using zebrafish embryos and the phenotypes screened include organ development,^[84,85] cell cycle control,^[86,87] and antiangiogenesis.^[88] Murphey *et al.* used developing zebrafish embryos to screen for cell cycle inhibitors. They screened a 16,320-compound library (DIVERSet) in 384 well plates and identified 14 compounds with confirmed novel activities in cell cycle control.^[87] Most recently, Tran *et al.* conducted an antiangiogenic compound screen using fluorescent transgenic zebrafish embryos *TG(VEGF2:GRCFP)*. They identified three compounds, SU4312, AG1478 and indirubin-3'-monoxime (IRO), as antiangiogenic compounds in zebrafish. IRO was further confirmed to inhibit human umbilical vein endothelial cell tube formation and proliferation.^[88]

8 Conclusion

Since George Streisinger and colleagues described the scientific value of the small (3–4 cm), striped teleost named *Danio rerio* about 28 years ago, the zebrafish has gradually become one of favorite model organisms for developmental biologists. The technological advances to functionally dissect the zebrafish genome by both forward and reverse genetics-based approaches allow the generation of mutant zebrafish strains at a tremendously increased pace, which will greatly facilitate the annotation of the vertebrate genome. The newly developed zebrafish gene targeting technology immediately places zebrafish in the frontier of modeling human diseases. It is also expected that the chemical genetic screening in zebrafish will lead to the discovery of novel therapeutic drugs to target many diseases and biological pathways.

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