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Use of Transgenic Fish Possessing Special Genes as Model Organisms and Potential Applications

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Recently zebrafish (Danio rerio) and medaka (Oryzias latipes) become the most useful model organisms since they are primitive vertebrates yet have many advantages over the other, such as light-induced spawning, easy transgenesis, transparent embryos, accessible mutagenesis, various mutants and complete genomic information. These advantages of model fish embryos are the most effective ways to map expression profiles, to dissect the regulatory cis-elements and to identify the gene functions of vertebral genes. Germ-line transmission is much more easily accessible. Transgenic line carrying a specific tissue tagged by fluorescent protein live marker becomes a powerful tool to study developmental biology because they enable to recapitulate the expression of endogenous genes. Furthermore, the transparent embryos are available for observation and tracing dynamic expression of a specific gene during embryogenesis. Transgenic fish can also serve as a simple animal model to study human diseases because defective phenotypes of some zebrafish mutants are similar to certain human diseases symptoms. Transgenic model fish are potentially applied as biosensors for detecting the environmental pollutants. In addition, the gene transfer technology can also be applied in commercial fish to enhance the quality or to improve the genetic characters of aquaculture broodstocks. The anti-freezing transgenic salmon and the fast-growing transgenic fish and shellfish have been reported and consider as the most successful cases until now. It highlights a new access to the modification of food fish and shellfish. However, the issues of genetically modified organisms (GMO) are still controversial. Food safety and ecological impacts are the major concerns before GMO are put in the market. Interestingly, new pet fish species that glows colorfully in the dark can be achieved by transferring fluorescent protein genes. Therefore, we conclude that transgenic fish is apt for modern biomedical researches and applications as new materials.

Key words: *Developmental biology, Genetically modified organisms, Germ-line transmission, Tissue-specific expression, Transgenic fish.*

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Introduction

Gene transfer technology is to introduce the foreign DNA fragments or RNA molecules into nucleus or cytoplasm of gametes, zygotes, embryos or somatic cells using physical or chemical approaches allowing foreign gene be reproduced and expressed in the host cells, before genetic messages are shown on individuals brought in by the transferred DNA or RNA. Those foreign DNA fragments may be originated from the host genome, related species or totally different species after genetic engineering. Such a DNA fragment can be cDNA or genomic DNA but at least it must consist of: (1) the regulatory regions, such as enhancer, repressor, promoter or initiator; (2) the coding region for the production of protein; and (3) the untranslated regions, including terminator. Only after such a transfer, a gene fragment would then make the protein perform actively inside the cell of the host. When fish is treated with this transfer technique, the fish would then feature nature and display the genetic traits encoded by foreign genes, making it known as transgenic fish.

The application of gene transfer on fish is a technology born a couple decades ago. Many species of transgenic fish have been obtained since the first transgenic goldfish was reported (Zhu *et al.*, 1985). Some finfish and shellfish species are key contributors of protein source to humans'food, gene transfer technology for aquatic animals may also be applied to enhance the quality or to improve the genetic characters of aquaculture broodstocks, although the issues of genetically modified organisms (GMO) are still controversial. On the other hand, for research purposes, zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are two major species that are considered as the most modern model organisms. In the molecular biology and embryology, fish is a primitive vertebrate yet has many advantages that other model systems do not have. For example, these model fish lay large quantity of eggs of large diameter. Ovulation is simply controlled by light under a 14-h light and 10-h dark regime. Spawning takes place frequently and year round. Microinjection of fertilized eggs is easily accessible. Maturation time is short, it takes 2~3 months before the eggs mature, indicating they are good materials to carry out germ-line transmission. Their embryos are transparent so that the pattern formation of the tissues and organs are easily observed without sacrificing individuals. The medaka and zebrafish genomes come in 20 and 40 % of the size that of mammals, respectively, and the only vertebrate available for large-scale mutagenesis. Naturally, model fish become an excellent material for studying vertebrate-specific genes and their functions.

Gene Transfer Systems

There are many systems that deal with introducing foreign DNA fragments into conceived fertilized embryos or gametes of aquatic species, to name a few: chemical additives (such as polyethylene glycol), microinjection (Chourrout *et al.*, 1986; Ozato *et al.*, 1986), electroporation (Inoue *et al.*, 1990), particle bombardment (Kolenikov *et al.*, 1990), sperm-mediated gene transfer (Muller *et al.,* 1992), retroviral infection (Lin *et al.*, 1994; Gaiano *et al.*, 1996), direct muscle injection (Tseng *et al.*, 1995; Xu *et al.*, 1999), and insulator and transponson (Caldovic and Hackett, 1995; Raz *et al.*, 1997),. Recently, nuclear transplantation is successfully developed by using embryonic-derived stem cells (Wakamatsu *et al.*, 2001), cell-mediated gene transfer (Ma *et al.*, 2001a) and zebrafish sperm (Jesuthasan and Subburaju, 2002). Cloned zebrafish has been also achieved (Lee *et al.*, 2002). Doubtlessly, cutting-edge techniques and modified methods have driven the study on fish gene transfer to an ever-improving horizon. At present, the most common techniques are microinjection, electroporation and sperm-mediated

gene transfer for aquatic animals.

1. **Microinjection:** the DNA solution is refilled into a micro-capillary glass needle having a pointed (stretched with a puller) and sharp opening. Thus, the glass needle is easily penetrated into zygotic or embryo cells of the fish just like a fine needle for the injection of 5~10 pL of the DNA solution with an injection manipulator. The location of injection is either cytoplasm (Chourrout *et al.*, 1986) or germinal vesicle (Ozato *et al*., 1986). With the fish eggs visible with naked eyes, microinjection turns out to be a much easier and efficient approach compared to the tools used for the transfer of mammal cells. First of all, as a 10-time magnification is enough for us to observe the eggs and to carry out microinjection, an ordinary microscope would be fine for the job, and no inverted microscope would be necessary. Secondly, as glass laminas or gel can be used to hold eggs for injecting, no holding manipulator would be required.

   Some transgenic food fish species have been reported by microinjection when transgenic technique began to apply on fish: rainbow trout (Chourrout *et al*., 1986), channel catfish (Dunham *et al*., 1987), salmon (Fletcher *et al*., 1988) and tilapia *Oreochromis niloticus* (Brem *et al*., 1988). The fertilized eggs of these commercially important species are opaque and thick chorion. Some techniques, such as injecting through micropyle (Shears *et al.*, 1992b), micro-surgery (Chourrout *et al*., 1986), and decomposing chorion with enzyme (pronase or trypsin) (Zhu *et al*., 1985; Hallerman *et al*., 1988), were reported to solve these drawbacks. However, these works may all make transfer works less efficient. On the other hand, microinjection is overwhelmingly useful for model fish since their embryos are transparent. All detailed microinjection procedures for medaka and zebrafish are described by Ozato *et al*. (1992) and Westerfield (1995), respectively. Nowadays, it becomes the most conventional, reliable and winning tool in studying molecular biology and embryology.
2. **Electroporation:** gametes or embryos are kept in a cuvette where cell membranes are permeabilized to allow DNA molecules to get in whenever short electrical pulses are employed. When the electrical pulses disappear and the cell membranes recover, some foreign DNA fragments may be remained in the gametes or embryos. The operation is easy and goes fast, making it possible to process over hundreds of fish oocytes or fertilized eggs in one shut (Inoue *et al.*, 1990). Electroporation on fertilized eggs has been considered as a means of mass gene transfer for model fish medaka (Inoue *et al.*, 1990) and many commercially important species such as catfish *Ictalurus punctatis*, common carp *Cyprinus carpio* (Powers *et al*., 1992), black porgy *Acanthopagrus schlegeli* (Tsai and Tseng., 1994), abalone *Haliotis rufescens* (Powers *et al*., 1995) and tiger shrimp *Penaeus monodon* (Tseng *et al*., 2000). The shortcoming is that the protocol of optimal condition is inconsistent, stage- and species-dependent, resulting in the poor successful rate and reproducibility. No way to know how many DNA molecules is in and where these DNA molecules have gone after electroporation. This is because foreign genes may be introduced into the gamete or embryo (including yolk sac) from all directions and they are not necessarily transferred to the animal pole, which is only one-tenth volume of eggs but where the exogenous DNA fragments have a chance to be integrated into host genome via recombination.
3. **Sperm-mediated gene transfer:** the gene transfer is conducted by sperm carrying foreign DNA. This transfer method, which has been applied on aquaculture species, has many advantages: (1) it is considered

as a mass gene transfer system; (2) sperm are easily prepared for transferring; (3) sperm can be kept by cryopreservation; (4) this system overcomes many drawbacks in using eggs as a vehicle, such as opaqueness, stickiness, buoyancy, tough chorion, and invisible pronuclei or micropyle. This system further improves the odds of getting valuable transgenic fish, especially when it comes to species of short spawning season, few frequency, and unclear developmental embryology, sperm-mediated gene transfer virtually become a winning means. Sperm-mediation gene system is a rapid and simple, yet practical approach than any other eggs-based transfer systems for commercially important species. Although transfer gene by sperm-incubation fails in rainbow trout (Chourrout and Perrot, 1992), many reports demonstrate that sperm-electroporation allow the exogenous DNA fragments be successfully transferred into the market fish and shellfish: common carp, African catfish, tilapia (Muller *et al.*, 1992), salmon (Sin *et al*., 1993), loach (Tsai *et al*., 1995a) and small Japanese abalone (Tsai *et al.*, 1997). A unique spermatophore-microinjection technology also successfully achieves gene transferring in giant freshwater prawn *Macrobrachium rosenbergii* (Li and Tsai, 2000). Recently, the method of direct testis-microinjection on abalone is also successfully developed by Chen *et al.* (2006).

Each transfer method has its pros and cons. Successful transfer is totally dependent on the accessibility and application of materials and equipments. We transferred foreign DNA fragments, in which the *lac*Z reporter gene is directed by the RSV-LTR promoter, into medaka embryos (Tsai *et al*., 1995b). No matter how many eggs were injected each time, around 30-40% of the injected embryos are turned out to appear positive signals at 12 hours-postfertilization, suggesting high reliability. In addition, when we microinject anti-sense morphoino-oligonucleotides (MO) to inhibit the translation of a specific gene via loss-of-function approach, the percentage of morphological defects remain perfectly reproducible (Chen and Tsai, 2002). On the other hand, we notice that the transgenic zebrafish developed by sperm-mediated gene transfer through sperm-incubation or sperm-electroporation, while transgenes have been transmitted to the progeny but those transgenes fail to express with unknown reason (Khoo *et al*., 1992; Patil and Khoo, 1996).

In the case only to get some useful transgenic fish having a special genetic trait out of a less accessible material with opaque embryos and limited spawning season, electroporation and sperm-mediated gene transfer are good alternatives for their being relatively easy and handy systems. Unlike sperm-mediation in zebrafish, after sperm-mediated gene transfer the transgenic GH gene is reported to enhance the growth rate in salmon (Sin *et al*., 1993; Symonds *et al*., 1994), loach (Tsai *et al.*, 1995a) and abalone (Tsai *et al*., 1997).

Germ-line Transmission

Although the foreign DNA fragment is successfully transferred in the embryos and then present in the G0 generation, a transgenic line has to be established to ensure the transgene is stably transmitted to the following generations without losing or any modification. Successful transfers for fish with microinjection or electroporation in unfertilized oocytes, one- , two- or four-cell stage are reported. Among them, stable lines of several transgenic fish species are generated (Stuart et al., 1990; Lu et al., 1992). Dunham *et al.* (1992) highlight their report that within 0- 52% of progeny produced by pairing transgenic and wild-type catfish carry transfer genes. Shears *et al.* (1992a) has proved that approximately 3% of Atlantic salmon progeny has been

detected transgenic gene in their blood and some 15-33% of progeny are transgenic fish when G0 transgenic salmon mating with wild-type. Hsiao *et al.* (2001) have also proved that on *actin*-GFP transgenic zebrafish transgene inheritance rates in F1α- and β-actin transgenic lines ranged 7-16% and 0.5- 49%, respectively. These results illustrate that transgenic founders may carry the transgene only in the portion of their cells due to the late integration of transgene in the host genome during early embryogenesis. Thus, not all transgenic founders can achieve successful germ-line transmission. The transfer gene must be integrated into the fish chromosomes before the first cell division of the fertilized eggs, so that it does exist in each and every later divided cell. Otherwise, transferring the gene is inserted into one of divided cells, only the derived organ or tissue would carry foreign genes, the so-called mosaicism. In other words, if the germ cells of founder fail to carry foreign gene, it would not transmit it onto the progeny as it would only be confined to parental performance. PCR and Southern blot analysis are the most common way to examine whether transgene exists in progeny. In general, around 10-20% of injected eggs may have germ-line transmission (Hackett, 1993). Hsiao *et al*. (2001) report that 44-59% of the F2 progeny of transgenic F1 males crossed with wild-type female exist transgene. The ratio of transgenic F3 progeny to non-transgenic progeny is 3:1. The ratios of heterozygotic and homozygotic progeny are in a Mendelian inheritance manner because the transgene is integrated in a single locus with a concatamerized multiplier.

Transgenic lines of food fish and shellfish that have been genetically improved have to be generated, particularly for broodstocks, since the genetic trait encoded by the transgene can surely be presented in every progeny. Transgenic line is also very important in using model fish to study the molecular and developmental biology because the transient expression patterns of integrated transgene in the G0 may be ectopic or highly mosaic, which is not faithful to the endogenous gene. In addition, transgenic lines are indispensable for studying the homozygotic lethal mutant genes and the targeted misexpression. In the latter system, an activator line and an effector line have to be generated (Scheer and Campos-Ortega, 1999).

New Materials for Developmental and Biomedical Studies

The gene transfer technology applied in model fish has been standardized. As compared to mice, chick and *Xenopus* systems, transgenesis on model fish is relatively easy, cheap and efficient to generate the transgenic lines that are able to feature some specific genes. These transgenic fish are generated by microinjecting a DNA construct in which a reporter gene driven by a tissue-specific promoter or/and enhancer. The transgenic fish lines can faithfully express the transgenic reporter gene as the endogenous gene presents. Although sometimes the transient expression assay of G0 embryos is mosaic, *in vivo* transient assay of zebrafish embryos is the most effective way to micro-dissect the regulatory *cis*-elements that control the tissue-specific expression of gene and to generate expression profiles of any desired deletion DNA fragments (Chen *et al*., 2001). On the other side, knock-down gene expression by MO is reliable and specific. In addition, mutagenesis is working well on zebrafish through ENU, radiation, proviral insertions and transposon insertions. Zebrafish mutants are more easily screened out and characterized (reviewed by Talbot and Hopkins, 2000).

Reporter gene is used to trance and/or quantify the transgene expression in the host organism and cell line. Chloramphenicol acetyltransferase, β-galactosidase andβ-lactamase are commonly used in zebrafish (Winkler *et al.*, 1991; Raz *et al*., 1998).

More recently, the green fluorescence protein (GFP) from jellyfish (*Aequorea victoria*) becomes the most popular reporter gene because GFP can produce fluorescence under fluorescent microscope *in vivo* without adding any substrate (Amsterdam *et al*., 1995). It is named live reporter gene. Establishing the germ-line transmission of a GFP reporter gene driven by regulatory elements of various sizes in zebrafish is a relatively simple and reliable procedure. Zebrafish gene promoters that drive tissue-specific GFP-expression in transgenic lines have been developed, including the erythroid-specific GATA promoter (Long *et al.*, 1997), muscle-specific α-actin promoter (Higashijima *et al.*, 1997), rod-specific *rhodopsin* promoter (Kennedy *et al*., 2001), neuron-specific *isl*-1 promoter (Higashijima *et al*., 2000), pancreas *pdx*-1 and *insulin* promoters (Huang *et al.*, 2001), myocardium-specific *cmlc*2 promoter (Huang *et al.*, 2003), liver-specific L-FABP promoter (Her *et al*., 2003), germ-cell-specific *vasa* promoter (Krovel and Olsen, 2002) and ovary-specific line (Hsiao and Tsai, 2003). In addition, uniform GFP expression throughout the whole body by using β-actin promoter is reported in medaka (Chou *et al*., 2001) and zebrafish (Higashijima *et al*., 1997; Hsiao *et al*., 2001). The upstream regulatory regions of above promoters are relatively short, ranged 0.5 to 6.5 kb, but they are sufficient to direct the reporter GFP to mimic the endogenous expression of genes.

In contrast, some genes should require an extremely long regulatory region (greater than 20 kb) to recapitulate the endogenous expression with less mosaic. Bacterial artificial chromosome (BAC, Jessen *et al*., 1998) and phage P1-dereived artificial chromosome (PAC, Jessen *et al*., 1999) are developed through homologous recombination to allow a large fragment fused with GFP be inserted into fish genome. Results show that PAC containing 80 kb of 5'and 40 kb of 3' sequences flanking the zebrafish *rag*1 drives a lymphoid cell-specific expression. More recently, instead of tedious procedure due to containing Chi site as reported by Jessen *et al.* (1998), a simpler two-step method to construct BAC is developed, which results in a BAC clone containing upstream 80 kb of 5'and 70 kb of 3'region of zebrafish *myf*5 and enables to mimic the endogenous *myf*-5 (Chen *et al*., 2007). To elucidate the nature of this finely tuned control mechanism of zebrafish *myf5*, Chen *et al.* (2007) generated a transgenic line that recapitulates the specific endogenous expression pattern of *myf5*. Such a transgenic line, Tg(*myf5*(80K):GFP) contains a 156-kb genomic sequence of *myf5* (upstream 80-kb and downstream 70-kb segments) replaced with green fluorescent protein (GFP) in the coding region. Several transgenic lines that carry various lengths of the zebrafish *myf5* upstream sequence were also generated (Chen *et al.,* 2007). All above transgenic fish lines are useful models for studying the regulation of gene expression and differentiation. Using germ-line transmission to map the upstream regulatory regions of a target gene may help solve the mosaic expression problem associated with G0 generations.

These advantages make the transgenic model fish apt for modern developmental and biomedical researches and applications as new materials (Grunwald and Eisen, 2002).

(1) As a model animal to study molecular and developmental biology. Zebrafish of gene transfer can be the most favorable material for research of vertebrate developmental biology (Udvadia and Linney, 2003). Usually, direct observation of the expression of genes in early developmental stage of mammal embryos in the womb is extremely difficult to access. Besides, it is almost impossible for researcher to use mice embryos to study the dynamic expression of a specific gene in different stages under *in vivo* condition. The specimen should be sacrificed to perform tissue sections, which

takes time and requires physical effort. Unlike mammal embryos, the transparent embryos of zebrafish are available for observation and tracing dynamic expression of a specific gene during embryogenesis, such as segmentation, gastrulation, neural patterning, axon guidance, embryonic induction, neurogenesis, organogenesis, sensory organ and cell signaling.

Heart development and angiogenesis. Tansgenic zebrafish is quite useful to study the development of heart because: (1) zebrafish comes with 1 atrium and 1 ventricle of heart, suggesting a primitive form of the heart of mammals that have 2 atria and 2 ventricles; (2) the heart is completely developed within 3 days after fertilization and the early development of heart is similar between fish and mammals, including cardiac primordial migration, heart cone and tube formation, jogging, looping, atrium and ventricle formation (Yelon and Stainnier, 1999); (3) the dynamic formation of heart can be easily traced due to the transgenic line that possesses the live GFP-tagged heart is available (Huang *et al*., 2003); (4) in the early stage of the embryo, it can survive for several days on diffused oxygen from water, even the cardiac and vessel system becomes defective, making the study on mutants in a defective heart possible; and (5) heart related mutants can be screened by a simple haploid mutation method or ENU. In addition, position cloning technology is available for screening out the putative genes and thereof functions (Stainier *et al*., 1996; Sehnert *et al.,* 2002). These advantages of model fish make researcher to discover genes involved in heart development become possible. For example, due to the zebrafish *jekyll* mutant, which is defective in heart valve formation, Walsh and Stainier (2001) discover that UDP-glucose dehydrogenase is required for cardiac valve formation. Due to the mutation of cardiac-specific sodium-calcium exchanger 1 (NCX1) gene, Langenbacher et al. (2005) found that NCX1 is responsible for rhythmical contraction of heart. Another example, due to mutation of cadherin 2 and plexin D1 genes and demonstrated that cadherin 2 and plexin D1 play important roles in heart and vessel patterning, respectively (Bagatto *et al.,* 2006; Torres-Vazquez *et al*., 2004).

We are the first lab to generate a transgenic zebrafish line that only has extremely strong fluorescence in the heart (Huang *et al*., 2003). This line is generated from embryos microinjected with DNA fragment containing a heart-specific regulatory segment of zebrafish cardiac myosin light chain 2 (*cmlc2*) fused with GFP cDNA. The expression of myocardium-specific GFP in this line can recapitulate the endogenous *cmlc2* gene (Huang *et al*., 2003). Interestingly, by using this transgenic line to study the heart regeneration, the Notch signal pathway is found to activate during the regenerative response, rather than redeployment of a cardiac development program (Raya *et al*., 2003). In addition, the distinct roles in patterning zebrafish heart between α1B1 and α2 isoforms of Na,K-ATPase are elucidated (Shu *et al*., 2003). Combination of confocal laser scanning microscopy and four-dimensional visualization, Forouhar *et al*. (2006) examined the movement of cells in the heart tube and the flow of blood through the heart and concluded that contradict peristalsis as a pumping mechanism in the embryonic heart. Recently, Lee *et al.* (2007) used this transgenic line and clearly demonstrated that glycogen synthase kinase 3α (GSK3α) is necessary in cardiomyocyte survival, whereas GSK3β plays important roles in modulating the left–right asymmetry and affecting heart positioning. Based on these research achievements, we believe this transgenic fish becomes a valuable organism for tracing the migration and fate specification of the cardiac cells.

Gene regulation network of zebrafish myogenic regulatory factor *myf5* gene. The function Myod or Myf5 is required for

the determination of skeletal myoblasts, whereas Myogenin controls the functions in the transition from a determined myoblast to a fully differentiated myotube (Arnold and Winter, 1998). The expression of *myf5* is stage- and somite-specific, which leads to *myf5* is regulated complicatedly but in a fine tune manner. However, we take the advantages of model fish by using *in vivo* transient assay, essential elements located at the upstream region that control the somite-specific expression of zebrafish *myf5* gene are characterized (Chen *et al*., 2001). For example, in the proximal *cis*-regulatory region, we found a novel *cis*-element located at -82/-62 is required for somite-specific expression of *myf5* (Chen *et al*., 2003). Interestingly, using the yeast one-hybrid assay, Lee *et al.* (2006) found that Forkhead box d3 (Foxd3), a well-known regulator in neural crest development, interacts directly with the -82/-62 cassette to control the somite-specific expression of *myf5*. When *pax3*-MO was microinjected, the expression of *foxd3* was down-regulated greatly in the morphants, and the expression of *myf5* was similar to that of the *foxd3* morphants. Co-microinjection of *foxd3* mRNA and *pax3*-MO greatly restored the expression of *myf5* in the somites and adaxial cells. Therefore, Lee *et al.* (2006) proposed that *pax3* induces *foxd3* expression, which then induces the expression of *myf5*.

In term of negatively regulatory mechanism of zebrafish *myf5* gene, Lin *et al.* (2004) found that intron 1 of zebrafish *myf5* contains a silencer that specifically represses the activity of *myf5*. Functional analysis of intron 1 showed a strong, negative, *cis*-regulatory element was located at +502/+835. Its function was orientation- and position-dependent. The repressive capability of this silencer was completely dependent on two core motifs, IE1 (+502/+527) and IE2 (+816/+835), and a 156-bp spanning sequence that lies between them. Hsu *et al.* (unpublished data) demonstrated that no protein-DNA complex was formed when the IE1 or IE2 segment and the nuclear extracts were used to perform electrophoretic mobility shift assay. A plasmid that contains a zebrafish *myf5* (-6300/-1) promoter, a luciferase reporter gene and a *myf5* +502/+835, was constructed and microinjected into one-celled embryos, results showed that intron I enabled to reduced the luciferase activity down to 20% of control in the mouse muscular cell line C2C12. When the sense RNA corresponding for +502/+835 was microinjected, the luciferase activity was also reduced down to 20% of the control. In addition, the intron I-mediated repression was promoter-specific because only *myf5* promoter activity was repressed, but did not for other promoters, such as *myod*, *β-actin* and cytomegalovirus promoter. Based on these evidences, we propose that the molecular mechanism of zebrafish *myf5* repression modulated by intron I is greatly controlled at RNA level, but neither at DNA level nor protein level. In the distal regulatory region of zebrafish *myf5* gene, we also demonstrated that there is a repressive *cis*-element located at -10/-6 kb, which is similar to the mouse repressor at -58/-8.8 kb (Chen *et al*., 2007).

Myf5 and Myod function in trunk and craniofacial muscles developments. To dynamically observe the progress of muscle development and to understand the function of myogenic regulatory factors during myogenesis, a zebrafish transgenic line Tg(α-actin:RFP) that carries a DNA construct in which the RFP reporter is driven by a zebrafish fast-muscle α-actin promoter was generated (Lin *et al.*, 2006). All skeletal muscles, including cranial muscles, of this transgenic Tg(α-actin:RFP) fish are tagged clearly by RFP that makes this line become an excellent experimental material for studying muscle development. Lin *et al.* (2006) showed that when *myf5*- or *myod*-MO was microinjected into zygotes from Tg(α-actin:RFP) fish, RFP signals were still

observed in the trunk. However, no trunk muscles were observed when embryos were co-microinjected with *myf5*- and *myod*-MO. These results clearly indicated that Myf5 and Myod function redundantly during trunk muscle development. On the other hand, Lin *et al.* (2006) found that Myf5 was required for the development of the obliques, lateral rectus, sternohyoideus, and all pharyngeal muscles, whereas Myod was required for the development of superior rectus, medial rectus, inferior rectus, lateral rectus, and the ventral pharyngeal muscles. *myod* mRNA did not rescue the loss of the cranial muscle caused by injecting *myf5*-MO, or *vice versa*, suggesting that the functions of Myf5 and Myod were not redundant in head paraxial mesoderm, a finding different from their functions in trunk myogenesis. Therefore, Myf5 and Myod modulate distinctly during carniofacial muscle development.

(2) As a simple animal model to study human diseases. Being the lowest vertebrate, zebrafish becomes an excellent material to study human diseases because some zebrafish mutants can faithfully phenocopy many human diseases (Dooley and Zon, 2000; Shin and Fishman, 2002). In addition, the essential genes and their regulatory *cis*-elements that control the formation and organization of tissues and organs, such as heart, eye, gut, kidney, blood and bone are conserved through vertebrate evolution (Garrity *et al*., 2002). For example, the syndromic deficiencies of mutated *tbx5*, a T-box transcription factor that is responsible for cell-type specification and morphogenesis, are well retained between fish and mammals. The phenotypes of zebrafish mutant, *gridlock*, resemble the coarctation of human heart disease (Zhong *et al.*, 2000). More importantly, fish develop tumors in all organs and the histopathology of such neoplasia often resembles that of human tumors (Stern and Zon, 2003; Goessling *et al.*, 2007). Thus, the idea that zebrafish is an excellent animal to serve as a cancer model system (Amatruda *et al.,* 2002) becomes more and more popular.

Huang *et al*. (2005) developed the heart-specific Tet-On system in zebrafish transgenic line. This line was generated by transferring plasmids in which the *cmlc2* promoter of zebrafish drove the reverse tetracycline- controlled transactivator expressing in myocardium, and where the GFP gene was preceded by a *tet*-operator. The photo-images of induced GFP in the F2 larvae showed that the GFP level was dose-responsive and the GFP induction was also tightly controlled by doxycycline in the heterozygotic F2 adult fish. This Tet-On system developed in zebrafish should provide a very useful material to study the gene functions of a specific tissue at the late developmental stage. For example, Ho *et al.* (unpublished data) generated a transgenic zebrafish in which cardiac troponin C was conditionally knocked down after addition of an inducer. It was found that some of the transgenic fish displayed inconsistent beating of the atrium and ventricle; this type of arrhythmia is very similar to human heart disease caused by incomplete atrio-ventricular block. This is the first time anywhere that arrhythmia can be seen in a living specimen with the naked eye (no heart monitor is needed). This transgenic strain can be used in the development and assessment of heart drugs, and for research on the molecular mechanism of drugs.

Therefore, transgenic zebrafish lines and mutants would provide abundant sources to study the possible mechanism involved in human diseases. Furthermore, some promising molecules used for therapeutic value and gene therapy may also be screened out through these transgenic lines.

(3) As a biosensor for environmental pollutants. Usually, existing tests of impacts on creatures by environmental pollutants (aromatic hydrocarbons, heavy metals and environmental estrogens) mainly focus on mortality rate or abnormality rate.

However, before the test species die or show any abnormality, the tissues or organs of the developing embryo were subjected to impacts by pollutants, only there were no methods or materials available that would allow us conduct direct observation and detection. Now, the transgenic fish having tissue-specific fluorescent markers are the most recommended material because this fluorescent zebrafish can be used to examine (1) whether pollutants are the cause to deformed genes during early development of embryos; (2) which pollutant affects which tissue-specific gene; and (3) which dosage of pollutant results in the damage level of developing genes. Thus, transgenic zebrafish has a high potential to be used as biosensors to detect the environmental toxicants (Amanumam *et al*., 2000) and mutagens (Amanumam *et al.*, 2002). Besides, Chen and Lu (1998) propose that *CYP1A*1-GFP transgenic medaka line can be used to detect the environmental xenobiotics and the double transgenic line derived from crossing between line *CYP1A*1-GFP (GFP driven by cytochrome P450 promoter) and line VG-Lux (Lux driven by vitellogenin promoter) can be used to detect the environmental xenoestrogenic compounds. Another example is Yabu *et al*. (2005), which reported that thalidomide induces the lack of VEGF receptor, neuropilin-1, and Flk synthesis, resulting in anti-angiogenesis occurring at the arteries and veins development in the fin and tail of zebrafish embryos. Wang *et al.* (2006) characterized the *cis*-regulatory elements of zebrafish keratin 18 gene and generated a transgenic line, Tg(k18(2.9):RFP), that possesses the *cis*-element which controls the skin-specific expression. When embryos derived from Tg(k18(2.9):RFP) adults were treated with arsenite, abnormal phenotypes were not noticeable under the light microscope. However, arsenic keratosis was visible in the epithelial cells under the fluorescent microscope. The additional advantages of small, abundant, rapid development and transparent eggs of zebrafish make this vertebrate become more accessible to study molecular toxicology.

Transgenic Fish and Shellfish for Industrial Applications

(1) Generating new pet fish species. Pet aquarium is believed as one of the best ways to make people relaxed from their day-to-day stress. When it comes to exploring ornamental fish, introduction and modification of various species is key to success. The traditional approaches to modification were mostly through culture of mutants or cross among species. Those processes take time without assuring to get ideal combinations of appearances. Things can change with molecular biotechnology, in which genetic engineering and gene transfer are two indispensable means. Fish body color can be multiplied or modified rapidly, making tropical fish more colorful or even glowing in the dark. Fluorescent transgenic medaka (Chou *et al*., 2001) and zebrafish (Hsiao *et al*., 2001) are good examples of genetically modified (GM) new strains that produce fluorescent color. Interestingly, the fluorescence appeared in these transgenic lines are so strong that green could be visually noticeable to the unaided eyes. Thus, lit by dark lights, fluorescent fish would emit fluorescence to drive pet aquarium in nighttime to a new horizon and allow people to enjoy the aquarium both daytime and nighttime spectacles. Besides, transgenic fish undergoing such a modification to have green/red/yellow could cross each other to generate new strains with mix colors (Gong *et al*., 2003). The fluorescent body colors are stable and permanent and do not change when the environment does or when they grow. Fish species would then be remarkably valued and competitive in the international market. TK-1, or "Night Pearl", a derivates of green fluorescent medaka, is the first GM pet in the world (Dean, 2003; Holden, 2003). Unlike

transgenic food fish, the only concern about marketing of GM pet fish is its ecological impact. Fortunately, more recently the technology of how to achieve 100% sterilization of Tk-1 progeny is successfully developed (Lee and Tsai, unpublished results). Thus, this GM pet fish should be considered as an environmental and ecological safety.

(2) Generating GM aquaculture fish and shellfish. Anti-freezing proteins and antifreeze glycoproteins are found in cold water marine teleosts such as winter flounder (*Pseudopleuronectes americanus*), shorten sculpin (*Myoxocephalus scorpinus*), sea raven (*Hemitripterus americanus*) and ocean pout (*Macrozoarces americanus*) (Hew *et al.*, 1988). Their major function is to keep the blood from freezing. After the DNA fragment encoding the type III antifreeze protein is transferred, Atlantic salmon (*Salmo salar*) starts showing the features in anti-freezing ability through the anti-freezing protein (Shears *et al.*, 1991).

While application of the recombinant GH to fish is a transient effect in aquaculture, gene transfer of GH gene is a technology with permanent effect. This is because the transferred GH gene is integrated into the host chromosome and is translated constitutively into GH polypeptide. The GH content is increased in the serum, making the fish specie grow faster. In this case, the gene transfer results in genetic modification that make the gene successfully transferred fish species feature foreign gene that has been inserted. Devlin *et al.* (1994) construct an expression plasmid containing an antifreezing protein promoter to drive salmon GH cDNA and transfer in to *S. salar*. The results show that a 6-fold greater growth in average weight of the transfer group as compared to that of control group. The largest salmon has undergone gene transfer gains weight by 76.7 g, almost 13 times that of the control group. Using the same gene construct, Tsai *et al.* (1995a) performed a sperm- electroporation for loach. The gene transfer rate is as high as 50%. The average weight gain in the experiment group for 4 months is 2.5 times higher than that of the mock group (with all treatments but DNA conducted on the experiment group) and the control group (without any treatment). Likewise, the transgenic abalone having the same construct grows just as fast as in G0 generation (Tsai *et al.*, 1997).

(3) Potential risk of GM aquaculture fish. It remains a key issue worth paying attention to the influences or impacts triggered by such an inlet transgenic species containing foreign natures on local ones that may jeopardize the ecosystem. Reichhardt (2000) indicated that GM fish is now in its mature stage and as of this day, several commercial food species have undergone successfully for fast-growing and anti-freezing nature. "Frankenfish"launched by the Canadian research team in 1995 is the renowned one with remarkable effect. It deals with transferring GH gene in *S. salar* for fast growth. Aqua Bounty Farms as filed application to FDA for a permit that would allow it to operate in the North American market, an attempt that has not been approved yet, as the company has filed to produce hard evidence showing that transgenic salmon does not cause negative impacts on the ecosystem.

Devlin *et al.* (1999) report that growth-enhanced transgenic coho salmon consumes much more feeds than wild-type strain, though its response is much slower than wild-type and more vulnerable to predators. It remains to be proved through field tests whether the GH-transgenic fish suffering from hungrier all the time would cause food competition with wild-type, and even consume food rationed to other species in food chain and destroy the ecosystem. At the same time, Muir and Howard (1999) find out that the transgenic medaka does grow faster than wild female and wins out in breeding. However, their offspring live shorter life span and is vulnerable to death before

becoming sexually mature. When the transgenic fish are kept with wild-type at the ratio of 1:1000, a mathematical computing model suggests that the original gene pool of wild-type would go extinct in 40 generations. This result clearly suggests that if transgenic fish were set free to the wild nature by accident, a race would be triggered against its wild rival, which might eventually be eliminated.

The most feasible approach is the production of sterile transgenic fish using chromosomal manipulation to keep foreign genes from being permitted into the gene pool of original species. In addition, facilities around the fish ponds, such as water flow, wastewater discharge or overflow, predators (people, domestic animals and birds) as well as boundary measures, shall be provided to keep transgenic fish from escaping. Naturally, culturing the transgenic fish in the cage at the open sea will increase the potential risks.

Conclusion

The research of the gene transfer technology applied in aquatic species (fish and shellfish) has not only improved the quality of commercially important species, but also generated the unique traits of model fish that are able to feature some specific genes. While we see more and more reports on successful transgenic fish cases, it becomes increasingly clear that transgenic fish is a potential material nobody should ignore when it comes to molecular biology and applied sciences. It provides a simple yet effective approach to molecular biology research to determine adjustment and function of vertebral genes *in vivo*. As for application, it highlights a new access to the modification of fish and shellfish. We can be sure that these transferred fish and shellfish exhibit the genetic features encoded by the transferred genes and other innate genetic characteristics remain as well, making themselves new strains. The new strains do not only display the unique character, they can transmit the transferred genes onto next generations in a stable manner. Compared to the conventional improvement of fish species by crossing, gene transfer is much easier yet effective system, if we know the function and regulation of target genes. Applied on aquaculture, genetic engineering and gene transfer can be cutting-edge technologies and significant boosts to the fishery industry. High transfer rate and stable germ-line transmission would be key objectives in future studies on gene transfer.

In general, modification of fish with genetic engineering is now in its mature stage and as of this day, a large number of species, including salmon, trout, catfish, tilapia and model fish, have undergone successful genetic transformation for fast-growing and anti-freezing nature. In the future, we anticipate many strains that have anti-cold, anti-saline, anti-pathogens, and meaty can then be raised to meet all environmental requirements for higher survival and lower cost, eventually the fishery industry would boom and provide more quality protein to mankind.

On the other hand, what we should be concerned about, when it comes to transgenic fishery, are: (1) No transgenic species should mean dramatic impacts on the ecosystem. “Trojan gene effect”as described by Muir and Howard (1999) should be completely avoided. For example, as new transgenic species of fast-growing might tend to dominate the environment and could undermine the ecosystem. In addition to legislation, we have to turn transgenic offspring sterile, as a way to protect the ecosystem. (2) The gene construct used to gene transfer shall originate from same species or similar nature (Du *et al.*, 1992) and no harmful genes shall be allowed, assuring that all animal species remain with their own gene pool and diversity. (3) A food safety assessment should be conducted for the transgenic

fish that are consumed. The materials carried and/or accompanied by the transgene should be safe and no unexpected proteins are produced due to the insertion sites of transgene.

References

Amanumam, K., H. Takeda, H. Amanumam and Y. Aoki (2000). Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. *Nat. Biotechnol*., **18**: 62-65.

Amanumam, K., S. Tone, H. Saito, T. Shigeoka and Y. Aoki (2002). Mutational spectra of benzo[a]pyrene and MelQx in rpsL transgenic zebrafish embryos. *Mutat. Res.*, **513**: 83-92.

Amatruda, I. F., J. L. Shepard, H. M. Stern and L. I. Zon (2002). Zebrafish as a cancer model system. Cancer Cell, **1:** 229-231.

Amsterdam, A., S. Lin and N. Hopkins (1995). The *Aequorea victoria* green fluorescent protein can be used as a reporter in live zebrafish embryos. *Dev. Biol.,* **171**: 123-129.

Bagatto, B., J. Francl, B. Liu and Q. Liu. (2006). Cadherin 2 (N-cadherin) plays an essential role in zebrafish cardiovascular development. *BMC Dev. Biol.,* **6:**23.

Brem, G., B. Brenig, G. Horstgen-Schwark and E. L. Winnacker (1988). Gene transfer in tilapia (*Oreochromis niloticus*). *Aquaculture,* **68:** 209-219.

Caldovic, L. and P. B. Hackett (1995). Development of position independent expression vectors and their transfer into transgenic fish. *Mol. Mar. Biol. Biotechnol.,* **4**: 51-61.

Chen, H. L., H. S. Yang, R. Huang and H. J. Tsai. (2006). Transfer of a foreign gene to Japanese abalone (*Haliotis diversicolor supertexta)* by direct testis-injection. *Aquaculture,* **253**:249-258.

Chen, Y. H., W. C. Lee, C. F. Liu and H. J. Tsai (2001). Molecular structure, dynamic expression and promoter analysis of zebrafish (*Danio rerio*) *myf-5* gene. *Genesis: J. Genet. Dev.,* **29:** 22-35.

Chen, T. T. and J. K. Lu (1998). Transgenic fish technology: basic principles and their application in basic and applied research. In *Gene Transfer in Aquatic Organism* (J. de la Fuente and F. O. Castro, eds.). Springer-Verlag, Berlin, 45-73.

Chen, Y. H., H. C. Lee, C. F. Liu, C. Y. Lin and H. J. Tsai (2003). Novel regulatory sequence -82/-62 functions as a key element to drive the somite-specificity of zebrafish *myf*-5. *Dev. Dyn.*, **228**: 41-50.

Chen, Y. H. and H. J. Tsai (2002). Treatment with Myf-5 morpholino results in somite patterning and brain formation defects in zebrafish. *Differentiation,* **70**: 447-456.

Chou, C. Y., L. S. Horng and H. J. Tsai (2001). Uniform GFP expression in transgenic medaka (*Oryzias latipes*) at the F0 generation. *Transgenic Res.,* **10**: 303-315.

Chourrout, D., R. Guyinard and L. M. Houdebine (1986). High efficiency gene transfer in rainbow trout (*Salmo gairdneri*) by microinjection into egg cytoplasm. *Aquaculture,* **51**: 143-150.

Chourrout, D. and E. Perrot (1992). No transgenic rainbow trout produced with sperm incubated with linear DNA. *Mol. Mar. Biol. Biotechnol. ,* **1:** 282-285.

Dean, J. (2003). Pets that glow: the future of bioengineered house pets is swimming in a tank of Taiwan. *Far Eastern Economic Review,* 38.

Devlin, R. H., T. Y. Yesaki, C. A. Biagi and E. M. Donaldson (1994). Extraordinary salmon growth. *Nature*, **371**: 209-210.

Du, S. J., Z. Gong, G. L. Fletcher, M. A. Shears, M. J. King, D. R. Idler and C. L. Hew. 1992. Growth enhancement in transgenic Atlantic salmon by use of an “all fish” chimeric growth hormone gene construct. *Bio/Technology,* **10:** 176-181.

Dooley, K. and L. I. Zon (2000). Zebrafish: a model system for the study of human disease. *Curr. Opin. Genet. Dev.,* **10:** 252-256.

Dunham, R. A., J. Cash, J. Askins and T. M. Towns (1987). Transfer of the metallothionein-human growth hormone fusion gene into channel catfish. *Trans. Am. Fish. Soc.,* **116:** 87-91.

Dunham, R. A., A. C. Ramboux, P. L. Duncan, M. Hayat, T. T. Chen, C. M. Lin, K. Kight, I. Gonzalez-Villasenor and D. A. Powers. 1992 Transfer, expression, and inheritance of salmonid growth hormone genes in channel catfish, *Ictalurus punctatusa,* and effects on performance traits. *Mol. Mar. Biol. Biotechnol.*, **1:** 380-389.

Fletcher, G. L., M. A. Shears, M. J. King, P. L. Davis and H. L. Hew (1988). Evidence for antifreeze protein gene transfer in Atlantic salmon (*Salmo salar*). *Can. J . Fish. Aquat.*

Sci., **45**: 352-357.

Forouhar, A. S., M. Liebling, A. Hickerson, A. Nasiraei-Moghaddam, H. J. Tsai, J. R. Hove, S. E. Fraser, M. E. Dickinson and M. Gharib. (2006). The embryonic vertebrate heart tube is a dynamic suction pump. *Science,* **312**: 751-753.

Gaiano, N., A. Amsterdam, K. Kawakame, M. Allende, T. Becker and N. Hopkins (1996). Insertional mutagenesis and rapid cloning of essential genes in zebrafish. *Nature,* **383**: 829-832.

Garrity, D. M., S. Childs and M. C. Fishman (2002).The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development,* **129**: 4635-4645.

Gong, Z., H. Wan, B. Ju, J. He, X. Wang and T. Yan (2003). Generation of living color transgenic zebrafish. In *Aquatic Genomics: Steps Toward a Great Future* (N. Shimizu, T. Aoki, I. Hirono and F. Takashima, eds.). Tokyo, Springer, 329-339.

Goessling, W., T. E. North and L. I. Zon (2007). New waves of discovery: modeling cancer in zebrafish. *J. Clin. Oncol.* **25**: 2473-2479.

Grunwald, D. J. and J. S. Eisen (2002). Headwaters of the zebrafish-- emergence of a new model vertebrate. *Nat. Rev.,* **3**: 717-724.

Hackett, P. B. (1993). The molecular biology of transgenic fish, In *Biochemistry and Molecular Biology of Fishes* (P. W. Hochachka and T. P. Mommsen, eds.). Amsterdam, Elsevier Science, pp. 207-240.

Hallerman, E, M., J. F. Schneider, M. Cross, A. J. Faras, P. B. Hackett, K. S. Guise and A. R. Kapuscinski (1998). Enzymatic dechorionation of goldfish, walleye, and Northern pike eggs. *Trans. Am. Fish. Soc.,* **117**: 456-460.

Hew, C. L., N. C. Wang, S. Joshi, G. L. Fletcher, G. K. Scott, P. H. Hayes, B. Buettner and P. L. Davies (1988). Multiple genes provide the basis for antifreeze protein diversity and dosage in the ocean pout, *Macrozoarces americanus*. *J. Biol. Chem.,* **263**: 12049-12055.

Her, G. M., Y. H. Yeh and J. L. Wu (2003). 435-bp liver regulatory sequence in the liver fatty acid binding protein (L-FABP) gene is sufficient to modulate liver regional expression in the transgenic zebrafish. *Dev. Dyn.,* **227**: 347-356.

Higashijima, S., Y. Hotta and H. Okamoto (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *J. Neurosci.,* **20:** 206-28.

Higashijima, S., H. Okamoto, N. Ueno, Y. Hotta and G. Eguchi (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev. Biol.,* **192:** 289-299.

Holden, C. (2003). Random samples: that special glow. *Science,* **300:** 1368.

Hsiao, C. D., F. J. Hsieh and H. J. Tsai (2001). Enhanced expression and stable transmission of transgenes flanked by inverted terminal repeats from adeno-associated virus in zebrafish. *Dev. Dyn.*, **220**: 323-336.

Hsiao, C. D. and H. J. Tsai (2003). Transgenic zebrafish with fluorescent germ cell: a useful tool to visualize germ cell proliferation and juvenile hermaphroditism *in vivo. Dev. Biol.*, **262**: 313-323.

Huang, C. J., T. S. Jou, Y. L. Ho, W. H. Lee, Y. T. Jeng, F. J. Hsieh and H. J. Tsai. (2005). Conditional expression of a myocardium-specific transgene in zebrafish transgenic lines. *Dev. Dyn*., **233**:1294-1303.

Huang C. J., C. T. Tu, C. D. Hsiao, F. J. Hsieh and H. J. Tsai (2003). Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev. Dyn.*, **228:** 30-40.

Huang, H., S. S. Vogel, N. Lin, D. A. Melton and S. Lin (2001). Analysis of pancreatic development in living transgenic zebrafish embryos. *Mol. Cell. Endocrinol.,* **177**: 117-124.

Inoue, K., S. Yamashita, J. I. Hata, S. Kabeno, S. Asada, E. Nagahisa, T. Fujita and T. S. Okada (1990). Electroporation as a new technique for producing transgenic fish. *Cell Differ. Dev.,* **29**: 123-128.

Jessen, J. R., A. Meng, R. J. McFarlane, B. H. Paw, L. I. Zon, G.R. Smith and S. Lin (1998). Modification of bacterial artificial chromosome through Chi-stimulated homologous recombination and its application in zebrafish transgenesis. *Proc. Natl. Acad. Sci.,* USA **95**: 5121-5126.

Jessen, J. R., C. E. Willett and S. Lin (1999). Artificial chromosome transgenesis reveals long-distance negative regulation of *rag*1 in

zebrafish embryo. *Nat. Genet.*, **23**: 15-16.

Jesuthasan, S. and S. Subburaju (2002). Gene transfer into zebrafish by sperm nuclear transplantation. *Dev. Biol.,* **242:** 88-95.

Langenbacher, A. D., Y. Dong, X. Shu, J. Choi, D. A. Nicoll, J. I. Goldhaber, K. D. Philipson, and J. N. Chen. (2005). Mutation in sodium-calcium exchanger1 causes cardiac fibrillation in zebrafish. *Proc. Natl. Acad. Sci. USA,* **102**: 17699-17704.

Kennedy, B.N., T. S. Vihtelic, L. Checkley, K. T. Vaughan and D. R. Hyde (2001). Isolation of a zebrafish rod opsin promoter to generate a transgenic zebrafish line expressing enhanced green fluorescent protein in rod photoreceptors. *J. Biol. Chem.,* **276**: 14037-14043.

Khoo, H. W., L. H. Ang, H. B. Lim and K. Y. Wong (1992). Sperm cells as vectors for introducing foreign DNA into zebrafish. *Aquaculture,* **107:** 1-19.

Kolenikov, V. A., A. A. Alimov, V. A. Barmintsev, A. O. Benyumov, I. A. Zelenina, A. M. Karsonov, R. Dzhabur and A. V. Zelenin (1990). High velocity mechanical injection of foreign DNA into fish eggs. *Genetika*, **26**: 2122-2126.

Krovel, A. V. and L. C. Olsen (2002) Expression of a *vas*::EGFP transgene in primordial germ cells of the zebrafish, *Mech. Dev.*, **116**: 141-150.

Lee, H. C., H. Y. Huang, C. Y. Lin, Y. H. Chen and H. J. Tsai. (2006). Foxd3 mediates zebrafish *myf*5 expression during early somitogenesis. *Dev. Biol.,* **290**:359-372.

Lee, H. C., J. N. Tsai, P. Y. Liao, W. Y. Tsai, K. Y. Lin, C. C. Chuang, C. K. Sun, W. C. Chang and H. J. Tsai (2007). Glycogen synthase kinase 3 alpha and 3 beta have distinct functions during cardiogenesis of zebrafish embryo. *BMC Dev. Biol.***, 7,** 93.

Lee, K. Y., H. Huang, B. Ju, Z. Yang and S. Lin (2002). Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat. Biotechnol.,* **20:** 795-799.

Li, S. S. and H. J. Tsai (2000). Transfer of foreign gene to giant freshwater prawn (*Macrobrachium rosenbergii*) by spermatophore- microinjection. *Mol. Reprod. Dev.*, **56**: 149-154.

Lin, C. Y., R. F. Yunag, H. C. Lee, W. T. Chen, Y. H. Chen and H. J. Tsai (2006). Myogenic regulatory factor Myf5 and Myod function distinctly during craniofacial myogenesis of zebrafish. *Dev. Biol.*, **299**: 594-608.

Lin, S., N. Gaiano, P. Culp, J. C. Burns, T. Friedman, J. K. Yee and N. Hopkins (1994). Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. *Science,* **265**: 666-668.

Long, Q., A. Meng, H. Wang, J. R. Jessen, M. J. Farrell and S. Lin (1997). GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development,* **124:** 4105-4111.

Lu, J. K., T. T. Chen, C. L. Chrisman, O. M. Andrisani and J. E. Dixon. (1992). Integration, expression, and germ-line transmission of foreign growth hormone genes in medaka (*Oryzias latipes*). *Mol. Mar. Biol. Biotechnol.*, **1**: 366-375.

Ma, C., L. Fan, R. Ganassin, N. Bols and P. Collodi (2001a). Production of zebrafish germ-line chimeras from embryo cell culture. *Proc. Natl. Acad. Sci.,* USA. **98**: 2461-2466.

Ma, G. C., T. M. Wang, C. Y. Su, Y. L. Wang, S. Chen and H. J. Tsai (2001b). Retina-specific *cis*-elements and their binding nuclear proteins of carp rhodopsin gene. *FEBS Lett.*, **508**: 265-271.

Muir, W. M. and R. D. Howard (1999). Possible ecological risks of transgenic organism release when transgene affect mating success: sexual selection and the Trojan gene hypothesis. *Proc. Natl. Acad. Sci. USA*, **96:** 13853-13856.

Muller, F., Z. Ivics, F. Erdelyi, T. Papp, L. Varadi, L. Horvatha, N. Maclean and L. Orban (1992). Introducing foreign genes in fish eggs by electroporated sperm as a carrier. *Mol. Mar. Biol. Biotechnol.,* **1**: 276-281.

Ozato, K., H. Kondoh, H. Inohara, T. Iwamatsu and Y. Wakamatsu (1986). Production of transgenic fish: introduction and expression of chicken δ-crystallin gene in medaka embryos. *Cell Differ.,* **19**: 237-244.

Patil, J. G. and H. W. Khoo (1996). Nuclear internalization of foreign DNA by zebrafish spermatozoa and its enhancement by electroporation. *J. Exp. Zoo.,* **274:** 121-129.

Powers, D. A., L. Hereford, T. Cole, T. T. Chen, C. M. Lin, K. Kight, K. Creech and R. Dunham (1992). A method for transferring genes into the gametes of zebrafish *Brachydanio rerio*, channel catfhish *Ictalurus puunctatus* and common carp *Cyprinus carpio*. *Mol. Mar. Biol. Biotechnol.,* **1**: 301-308.

Powers, D. A., V. L. Kirby, T. Cole and L.

Hereford (1995). Electroporation as an effective means of introducing DNA into abalone (*Haliotis rufescens*) embryos. *Mol. Mar. Biol. Biotechnol.*, **4**: 369-376.

Raya, A., C. M. Koth, D. Buscher, Y. Kawakami, T. Itoh, R. M. Raya, G. Sternik, H. J. Tsai, C. Rodriguez-Esteban and J. C. Izpisua-Belmonte (2003). Activation of Notch signaling pathway precedes heart regeneration in zebrafish. *Proc. Natl. Acad. Sci. USA,* **100**: 11889-11895.

Raz, E., H. van Luenen, B. Schaerringer, R. Plasterk and W. Driever (1997). Transposition of the nematode *Caenorhabditis elegans Tc3* element in the zebrafish *Danio rerio*. *Cur. Biol*. **8:** 82-88.

Raz, E., G. Zlokarnik, R. Y. Tsien and W. Driever (1998). β-Lactamase as marker for gene expression in live zebrafish embryos. *Dev. Biol.,* **203**: 290-294.

Reichhardt**,** T. (2000). Will souped up salmon sink or swim? *Nature*, **406:** 10-12.

Scheer, N. and J. A. Campos-Ortega (1999). Use of the GAL4-UAS technique for targeted gene expression in zebrafish. *Mech. Dev.,* **80:** 153-158.

Shears, M. A., G. L. Fletcher, C. L. Hew, S. Gauthier and P. L. Davies. (1992a). Transfer, expression, and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). *Mol. Mar. Biol. Biotechnol.,* **1**: 58-63.

Shears, M. A., M. J. Kings, S. V. Goddard and G. L. Fletcher (1992b). Gene transfer in salmonids by injection through the micropyle. In *Transgenic Fish* (C. L. Hew and G. L. Fletcher, eds.). Singapore, World Scientific, 44-60.

Sehnert, A., A. Huq, B. M. Weistein, C. Walker, M. Fishman and D. Stainier. (2002). Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. *Nat. Genet.* **31**: 106-110.

Shin, J. T. and M. C. Fishman (2002). From zebrafish to human: modular medical models. *Annu. Rev. Genomics. Hum. Genet.,* **3**: 311-340.

Shu, X., K. Cheng, N. Patel, F. Chen, E. Joseph, H. J. Tsai and J. Chen (2003). Na,K-ATPase is essential for embryonic heart development in the zebrafish. *Development,* **130:** 6165-6173.

Sin, F. Y. T., A.L. Bartley, S. P. Walker, J. L. Sin, J. E. Symonds, L. Hawke and C. L. Hopkins (1993). Gene transfer in chinook salmon *(Oncorhynchus tshawytscha)* by electroporating sperm in the presence of pRSV-*lacZ* DNA. *Aquaculture,* **117**: 57-69.

Stainier, D. Y., B. Fouquet, J. N. Chen, K. S. Warren, B. M., *et al.* (1996).Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryos. *Development,* **123**: 285-292.

Stern, H.M. and Zon, L.I. (2003). Cancer genetics and drug discovery in the zebrafish. *Nat. Rev. Cancer,* **3**: 533-539.

Stuart, G. W., J. R. Vielkind, J. V. McMuttay and M. Westerfield. (1990). Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. *Development,* **109**: 577-584.

Symonds, J. E., S. P. Walkwer, F. Y. T. Sin and I. Sin (1994). Development of a mass gene transfer method in Chinook salmon : optimization of gene transfer by electroporated sperm. *Mol. Mar. Biol. Biotechnol.,* **3**: 104-111.

Talbot, W. S. and H. Hopkins (2000). Zebrafish mutations and functional analysis of the vertebrate genome. *Genes Dev.,* **14**: 755-762.

Torres-Vázquez, J., A. D. Gitler, S. D., Fraser, J. D., Berk, N. P. Van, M. S. Fishman, S. Childs, J. A. Epstein and B. M. Weinstein. (2004). Semaphorin-plexin signaling guides patterning of the developing vasculature. *Dev. Cell*, **7**:117-123.

Tsai, H. J., C. H. Lai and H. S. Yang (1997). Sperm as a carrier to introduce an exogenous DNA fragment into the oocyte of Japanese abalone (*Haliotis disvorsicolor suportexta*). *Transgenic Res.*, **6**: 85-95.

Tsai, H. J. and F. S. Tseng (1994). Electroporation of a foreign gene into black porgy (*Acanthopagrus schlegeli*) embryos. *Fish. Sci.*, **60**: 787-789.

Tsai, H. J., F. S. Tseng and I. C. Liao. (1995a). Electroporation of sperm to introduce foreign DNA into the genome of loach (*Misgurnus anguillicaudatus*). *Can. J. Fish. Aquat. Sci.,* **52**: 776-787.

Tsai, H. J., S. H. Wang, K. Inoue, S. Takagi, M, Kimura, Y. Wakamatsu, and K. Ozato (1995b). Initiation of the transgenic *lacZ* gene expression in medaka (*Oryzias latipes*) embryos. *Mol. Mar. Biol. Biotechnol.,* **4**: 1-9.

Tseng, F. S., I. C. Liao and H. J. Tsai. (1995). Transient expression of mouse tyrosinase gene in catfish albino mutants (*Clarias fuscus*) by subcutaneous microinjection.

Fish. Sci., **61**: 163.

Tseng, F. S., H. J. Tsai, I. C. Liao and Y. L. Song (2000). Introducing foreign DNA into tiger shrimp (*Penacus monodon*) by electroporation. *Theriogenology,* **54:** 1421-1432.

Udvadia, A. J. and E. Linney (2003). Windows in to development: historic, current, and future perspectives on transgenic zebrafish. *Dev. Biol.,* **256**: 1-17.

Wakamatsu, Y., I. Pristyaznhyuk, K. Niwa, T. Ladygune, M. Kinoshita, K. Araki and K. Ozato (2001). Fertile and diploid nuclear transplants derived from embryonic cells of a small laboratory fish, medaka (*Oryzias latipes*). *Proc. Natl. Acad. Sci. USA,* **98**: 1071-1076.

Walsh, E. C. and D. Y. R. Stainier (2001). UDP-Glucose dehydrogenase required for cardiac valve formation in zebrafish. *Science,* **293**: 1670-1673.

Wang, Y. H., Y. H. Chen, T. N. Wu and H. J. Tsai. (2006b). A keratin 18 transgenic zebrafish Tg(k18(2.9):RFP) treated with inorganic arsenite reveals visible overproliferation of epithelial cells. *Toxicol. Lett*., **163**:191-197.

Westerfield, M. W. (1995). The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). University of Oregon Press, Eugene, Oregon, USA.

Winkler, C., J. R. Vielkind and M. Schartl (1991). Transient expression of foreign DNA during embryonic and larval development of the medaka fish (*Oryzias latipes*). *Mol. Gen. Genet.,* **226**: 123-140.

Xu, Y., J. He, H. L. Chan, J. Liao, T. Yan, T. J. Lam and Z. Gong (1999). Fast skeletal muscle-specific expression of a zebrafish myosin light chain 2 gene and characterization of its promoter by direct injection into skeletal muscle. *DNA Cell Biol.*, **18**: 85-95.

Yabu, T., H. Tominioto, Y. Taguchi, S. Yamaoka, Y. Igarashiand and T. Okazaki. (2005). Thalidomide-induced antiangiogenic action is mediated by ceramide through depletion of VEGF receptors, and is antagonized by sphingosine-1-phosphate. *Blood,* **106**:125-134.

Yelon, D. and Stainier, D. Y. (1999). Patterning during organogenesis: genetic analysis of cardiac chamber formation. *Semin Cell Dev. Biol.*, **10**: 93-98.

Zhong, T. P., M. Rosenberg, M. A. Mohideen, B. Weinstein and M. C. Fishman. (2000). *gridlock*, an HLH gene required for assembly of the aorta in zebrafish. *Science*, **287**: 1820-1824

Zhu, Z., G. Li, L. He and S. Chen (1985). Novel gene transfer into the fertilized eggs of gold fish (*Carassius auratus* L. 1758). *Z. Angew. Ichthyol.,* **1**: 31-34.